ELECTRON MICROSCOPY OF TRYPSIN-MODIFIED AVIAN INFECTIOUS BRONCHITIS VIRUS INTERACTIONS WITH CHICKEN ERYTHROCYTES

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ABSTRACT

ELECTRON MICROSCOPY OF TRYPSIN-MODIFIED AVIAN INFECTIOUS BRONCHITIS VIRUS INTERACTIONS WITH CHICKEN ERYTHROCYTES

By

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Avian Infectious Bronchitis Virus (IBV) was harvested from infected chicken allantoic fluid and subjected to various partial purification techniques. Virion structural integrity was maintained during various conditions of temperature and preparation. If necessary, surface projections could be successfully secured to virions by mild fixation with glutaraldehyde at host temperatures.

Trypsin appears to affect partially purified preparations of IBV. Greater numbers of "dark center" virions were observed in treated samples than in untreated samples.

Partially purified samples of IBV which had been trypsinized caused hemagglutination (HA) to occur. Samples recovered from HA patterns contained virus-like particles attached to chicken erythrocytes. Nontreated samples also exhibited attachment, although to a far lesser extent, which was probably caused by the fixatives employed for electron microscopy.

Immobilizing virus-erythrocyte interaction in ionagar prevented non-specific bonds by glutaraldehyde. Virus-like particles in ionagar were seen in bridges annexing adjacent erythrocytes.

Hemagglutination may result from a multitude of factors, all possibly interrelated. Hemagglutinins could be chemically and/or physically monitored virions, modified or nonmodified extraneous allantoic fluid material, or both.

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DEDICATION

To Candy,

Thank you for your confidence,
devotion and contributions

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INTRODUCTION

Direct hemagglutination by Avian Infectious Bronchitis Virus (IBV) occurs only after infected chicken embryo allantoic fluid is treated with trypsin, other, or fractionated by diethylaminoethyl-column chromatography.

The effects of trypsin on IBV have been subject to dispute. This investigation possibly has resolved that dispute, in addition to attempting to elucidate the hemagglutinin-erythrocyte interaction by electron microscopy.

LITERATURE REVIEW

Avian Infectious Bronchitis Virus

Avian infectious bronchitis virus (IBV) is a highly contagious viral respiratory disease of chickens. First recognized as a distinct clinical entity in 1931 (Schalk and Hawn), the virus was isolated seven years later (Beaudette and Hudson, 1937).

Transmission of the virus is via aerosols from infected to cohabiting susceptible birds (Levine and Hofstad, 1947). Chickens of all ages, sexes and breeds are susceptible. The disease manifests itself as a general respiratory distress, with tracheal râles. There is a precipitous drop in egg production in the laying flock and a high mortality rate in young chicks (McDougall, 1968).

Although clinical symptoms are related to respiratory ailments, there is a viremic phase of the disease resulting in infection of the kidney, pancreas, spleen, liver, bursa of Fabricius and oviduct. Nephrosis and uremia (Cummings, 1963) may be sequela of infection.

Diagnosis may encompass clinical symptoms and isolation of the virus primarily from respiratory tissue using the chicken embryo as the cultural medium. Other

diagnostic tools such as neutralization (Fontaine *et al.*, 1963), immunodiffusion (Woernle, 1966), immunofluorescence (Lucio and Hitchner, 1970), direct (Brumfield and Pomeroy, 1957) and indirect (Steele and Luginbuhl, 1964) complement fixation, and indirect hemagglutination (Brown *et al.*, 1962) have also been employed. Due to modification of the virion, direct hemagglutination tests employing trypsin-treated virus (Corbo and Cunningham, 1959) are not commonly used.

Negatively stained virions are pleomorphic with an over-all diameter from 80 nm to 120 nm. The bulbous surface projections are approximately 9 nm to 11 nm in diameter on the distal end, and are attached to the virion by a narrow neck.

The internal structure of IBV has not been elucidated. However, ultra-thin sections of pelleted fresh infected allantoic fluid have revealed virions with a three-layered envelope 4 nm in width. Bulbous projections were not observed, although particle extensions of the outermost membrane layer were seen (Apostolov *et al.*, 1970). Conflicting results as to the possible existence of an electron-lucent space between the inner and outer membranes (Nazerian and Cunningham, 1968; Uppal and Chu, 1970) have also been reported.

The density of the Beaudette strain of IBV from allantoic fluid is 1.23, sedimentation 334S. By isopycnic density gradient centrifugation in caesium chloride, a

value of 1.24 was determined for the same strain propagated in Chicken Embryo Kidney Cells (CEKC) (Ellis, 1965). Other buoyant density values ranging from 1.16 to 1.27 (Tevethia and Cunningham, 1968; Mengeling and Coria, 1972; Tannock, 1973) may be attributed to differences in cultural media, menstrua, and/or pleomorphism of the virion.

The genome is composed of RNA as determined by fluorescence microscopy with acridine orange, susceptibility of the virus to RNase (Akers, 1963), and the use of analogs such as 5-bromouracil and 6-azauracil. The RNA has a molecular weight of 9.0×10^6 daltons and a sedimentation value of 50S (Watkins *et al.*, 1974). The nucleic acid is single stranded and discontinuous, exhibiting extreme heterogeneity in fragment size. Two classes of fragments are present, a larger class comprising 74.9-85.4% of the total RNA, with a molecular weight varying from 0.5×10^6 to 3.0×10^6 daltons, and a smaller class composed of 9.1-19.7% of the total RNA, with an appropriate molecular weight near 4S RNA (Tannock, 1973).

Sensitivity to ether (Petek and Corazzola, 1958), chloroform (Estola, 1966), sodium dodecyl sulfate (Berry *et al.*, 1964), and sodium deoxycholate (Tevethia and Cunningham, 1968) indicates the structural and functional need for lipids within the virion. Ether (Nazerian and Cunningham, 1967) and sodium dodecyl sulfate (Berry *et al.*, 1964) remove the surface projections from the virion and split the membrane with the consequential release of

internal components. However, little or no organized internal structures were discerned by electron microscopy.

Most strains of IBV harvested from the allantoic fluid of chicken embryos are inactivated at 56°C in 15 minutes (Hofstad, 1956), but others survive for as long as 160 minutes (von Bülow, 1967). Different experimental conditions, purity of virus preparation, and/or the presence of ions or colloids are contributing factors in this discrepancy.

Stability at acid pH differs among isolates of IBV. It is possible that this is a reflection of experimental conditions rather than viral characteristics (Cowen and Hitchner, 1975).

Intriguing and, indeed, one of the most perplexing problems in the study of IBV has been its fastidiousness in isolation and cultivation. The virus must first be isolated in, and adapted to, the chicken embryo before it is capable of growth in avian cells and organ cultures. A consequence of this transition is a decrease in immunogenicity, a probable result of spontaneous mutation caused by the fragmented genome.

While the chicken embryo is the most sensitive cultural medium (Lukert, 1965), it lacks the objectivity of typical cytopathic effects and the formation of plaques in CEKC or chicken kidney cell cultures.

There is a direct relationship between the amount of viral inoculum and replication (Becker, 1967), which

occurs entirely within the cytoplasm. Viral particles form within cytoplasmic vesicles and emerge from the cell via cytoplasmic tubules without budding through the cell membrane (Nazerian and Cunningham, 1968).

Infectious bronchitis virus possesses at least three antigenically distinct, non-infectious, nucleoprotein, soluble antigens detectable by immunodiffusion. Disruption of the virion by ether (Tevethia, 1964) releases the internal antigens. Antibody induced by these antigens does not neutralize viral infectivity.

The intact virion containing internal and surface antigens is responsible for the production of antibodies capable of neutralizing infectivity. The neutralization test is the only serologic test currently capable of differentiating between the various strains of IBV (Cunningham, 1973).

Electron microscopy has revealed that antibody in heated homotypic (chicken) antiserum attaches solely to the virion's surface projections. Unheated homotypic antiserum, or heat-labile antibody, forms a halo around the virion. Heterotypic (rabbit) antiserum contains antibodies against both surface projections and the viral envelope, and forms holes of approximately 10 nm in diameter in the virion (Berry and Almeida, 1968).

Infectious bronchitis virus was initially considered to be associated with the myxoviruses on the basis of certain common physical and biological properties. By

means of electron microscopy, bulbous projections seen on IBV were entirely different from the rod-like projections observed on myxoviruses (Berry *et al.*, 1964). This morphological differentiation of IBV was the sole criterion used for subsequent taxonomic classification of the coronaviruses.

The discovery of two human viruses, human strain 229E (Hamre and Procknow, 1966) and human coronavirus B814 (Almeida and Tyrrell, 1967), containing bulbous-like projections, led to the formation of a new viral family, the coronaviruses, of which IBV is considered the prototype (coronaviruses a-1) (Melnick, 1972).

Common properties of the coronaviruses are: 1) RNA genome; 2) lipid solvent labile; 3) 70-120 nm in diameter; 4) bulbous-tipped surface projections 15-20 nm in length; 5) plemorphic conformation; 6) nucleocapsid develops in the cytoplasm and matures in cytoplasmic vesicles.

Viral Hemagglutination

Subsequent to the initial discovery that influenza viruses agglutinate fowl erythrocytes (Hirst, 1941; McClelland and Hare, 1941), a number of different viruses have also been discovered to cause hemagglutination. The mechanism, however, may not be the same.

Evidence that viral particles per se or their soluble components are responsible for hemagglutination is the specific inhibition (hemagglutination-inhibition, HI) of

the reaction by viral antibodies. Human and animal sera also contain nonspecific inhibitors of hemagglutination in quantity to effectively compete with these specific antibodies (Rosen, 1960a).

The phenomenon, observed as a lattice pattern at the bottom of a test tube, serves as an assay for intact viral particles or their hemagglutinating subunits. The stability of the pattern varies inversely with the ability of the virus to elute (Salk, 1944).

The ability of viruses to agglutinate certain erythrocytes is commonly, although erroneously, referred to as a viral hemagglutinin. For convenience, however, this designation will be used in the future.

The reaction of myxoviruses with red blood cells involves two relatively independent phenomena: adsorption via a viral hemagglutinin (Webster, 1970), and elution via a viral neuraminidase (Wrigley *et al.*, 1973).

Virions disrupted by ether or detergents, and fractionated by electrophoresis or column chromatography (Laver and Valentine, 1969), have been shown via electron microscopy with negative staining, to contain hemagglutinin and neuraminidase as separate spikes on the envelope (Rott *et al.*, 1970). The isolated hemagglutinin is reactive with chicken erythrocytes, and appears in two different forms after negative staining. The first was a rod-like structure 5 nm in diameter and 20 nm in length.

The second form was composed of spherical particles 4 nm in diameter.

Adsorption is relatively independent of temperature, reacting equally well at 4°C and at 37°C, and is predominantly an electrostatic interaction with upper (pH 9.0) and lower (pH 5.0) pH limits (Tischer, 1963).

Myxoviruses have variable affinities for erythrocyte receptors. Elution is caused by RBC receptor destruction and the consequential release of N-acetyl neuraminic acid (NANA) by viral neuraminidase at 37°C (Hirst, 1942), and is inversely proportional to its sensitivity to glycoprotein hemagglutinating inhibitors (Choppin and Tamm, 1960), such as human and animal sera, egg components, urinary mucoproteins, ovarian cyst fluid, collocalia mucoid, and tissue extracts derived from several species (Howe *et al.*, 1961).

A soluble viral receptor (VRS) extracted from erythrocyte stroma with 50% hot phenol (Kathan *et al.*, 1961) is a sialoglycoprotein with an apparent molecular weight of 5.5×10^4 daltons (Springer *et al.*, 1966).

Reduced electrophoretic mobility of erythrocytes, and inability to re-agglutinate in the presence of fresh virus, have been observed after elution of influenza virus (Hanig, 1948). Treatment of the RBC with the receptor-destroying enzyme (neuraminidase - RDE) of *Vibrio (V.) cholera*, trypsin, or periodate, produces the same results.

Paramyxoviruses agglutinate both avian and some mammalian erythrocyte species, and in many respects parallel the myxoviruses. The viral hemagglutinin is situated on the viral spikes, distinct from, but in close association with, viral neuraminidase. However, the paramyxoviruses elute from the RBC with lower efficiency (Kisseljov *et al.*, 1969).

Erythrocyte receptors for both viral families are the same, as demonstrated by either family's mutual destruction of the RBC receptor. Trypsin and RDE, *V. cholera*, also destroy either RBC receptor.

Measles virus differs from the other paramyxoviruses in that it alone contains a hemagglutinin, but no neuraminidase (Waterson, 1965). The hemagglutinin is part of the viral envelope, and can be effective in the form of subunits or fragments of the envelope. It manifests itself in two forms: a large, infective or noninfective hemagglutinin, and a small hemagglutinin. The former has been characterized as either an intact infective particle, or one which has undergone some structural disorganization, rendered incapable of infectivity yet capable of hemagglutinating. The latter is noninfective, has a RNP core, and a variable density.

Trypsin inactivates the hemagglutinin, suggesting its protein-like structure. The measles virus RBC receptors are different in that they are not the same as the mutual

one for myxoviruses and other paramyxoviruses (Peries and Chany, 1962).

All serotypes of human adenoviruses (Bauer and Wigand, 1963; Norby, 1969; Rosen, 1958; Rosen, 1960a; Rosen *et al.*, 1962) are divided into three major groups depending upon their ability to agglutinate or partially agglutinate rat, rhesus, or human erythrocytes.

The complete hemagglutinins are the intact virions, with the distal knob of the fiber responsible for viral attachment, and several soluble structural forms of the virus such as dodecon, and dimers of either penton or fiber structure. Incomplete hemagglutinins are single pentons or fibers which, although monovalent, are still capable of bonding to RBC (Wadell, 1969), and can be rendered bivalent by complexing with heterospecific antibody (Norby, 1969).

Erythrocyte receptors have not been defined biochemically, although they have been solubilized and partially purified (Neurath *et al.*, 1969). The receptor material is not sensitive to neuraminidase and will not bind with myxoviruses. An exception is adenovirus type 9, subgroup II, which can selectively be removed from the RBC with neuraminidase (Wadell, 1969).

Inactivation of the hemagglutinin by trypsin, chymotrypsin, papain, pronase, and fiein suggests its proteinaceous nature.

Hemagglutination by many, particularly the Japanese B encephalitis viruses, but not all of the arboviruses has been demonstrated (Sabin and Buescher, 1950).

Environmental conditions for classification by hemagglutination are critical. Most group A arboviruses require an optimal 37°C at pH 6.4, while group B requires 4°C through 22°C at pH 7 (Cassals and Brown, 1954).

Use of various lipoprotein inhibitors (Clark and Cassals, 1958) has proven the lipoprotein nature of the virus-cell interaction.

Reoviruses are separated into three distinct serotypes by the HI reaction (Rosen, 1960b). Human erythrocytes are agglutinated by all three serotypes; however, type 3 reacts with bovine RBC with a higher titer. The reaction is neither temperature nor pH dependent, occurring equally well within 4°C to 23°C and pH 6 to 8. Elution by the virus from the erythrocyte is variable and nonenzymatic in nature.

Human erythrocyte receptors for reoviruses are not affected by neuraminidase *V. cholera*; however, bovine erythrocyte receptors for type 3 are sensitive (Eggers *et al.*, 1962). The hemagglutinin is sensitive to trypsin and chymotrypsin, and can be present in both complete and incomplete, or coreless, particles (Papadimitriou, 1966) for which -SH groups on the virion are essential.

Among the picornaviruses which possess HA activity, the ECHO and coxsackie B3 viruses have the greatest

suitability to human type O erythrocytes (Goldfield *et al.*, 1957). Reactions are possible under a wide range of temperature and pH conditions.

Elution of ECHO virus from the RBC is without effect on the cellular receptor to which fresh virus can be attached. Non-eluting particles can be removed by chymotrypsin during the first stage of adsorption. The lipoprotein (Philipson *et al.*, 1964) cellular substrate for some picornaviruses is not the same, although certain properties, such as sensitivity to RDE (Stott and Killington, 1972; Verlinde and De Baan, 1949) may be shared.

The hemagglutinating property resides in the capsid protein since both complete and incomplete viral particles are antigenically and reactively identical. Soluble purified erythrocyte receptors, after irreversible binding by ECHO 7, cause the release of infective viral RNA from the virion (Philipson and Lind, 1964).

Due to their newly perceived existence, little is known about the physical or chemical properties of the coronaviruses, particularly their ability, or lack thereof, to hemagglutinate. The human strains OC 38 and OC 43 (Kaye and Dowdle, 1969), which were adapted to growth in suckling mouse brain and are serologically identical, and Hemagglutinating Encephalytic Virus (HEV) (Phillip *et al.*, 1971) are the only coronaviruses which agglutinate erythrocytes without any treatment of virus. The human

strains agglutinate human type O and vervet monkey erythrocytes at 4°C only, whereas chicken, rat, and mouse cells react at any temperature. Hemagglutinating encephalytic virus agglutinates rat, chicken, turkey, mouse, and hamster erythrocytes at 22°C. After treatment with ether, neither the OC nor HEV strains agglutinate erythrocytes. Trypsin destroys the hemagglutinating activity of the HEV.

The inability to free the hemagglutinin of OC 43 by various chemical and physical treatments suggests that it is either closely associated with, or is an integral part of, the virion (Kaye *et al.*, 1970). However, bromelin (Hierholzer *et al.*, 1972) removes both the club-shaped projections and the hemagglutinating activity.

Hemagglutination by the two human strains was inhibited by specific antibody and the erythrocytes were unaffected by neuraminidase.

Trypsin (Corbo and Cunningham, 1959) or ether treatment (Biswal, 1965) of crude allantoic fluid from IBV-infected eggs induces HA activity. Diethylaminoethyl (DEAE) column chromatography of untreated infected allantoic fluid selectively separates the nonspecific inhibitor from the hemagglutinin, which is noninfectious, 60-70 nm in diameter, selectively separable from infectious virus, does not sediment at 136,000 x g for 2 hours, and contains a lipoprotein (34.2% lipid, 51.2% protein), with trace amounts of carbohydrate and RNA, but no detectable DNA. The noninfectious hemagglutinin destroys the RBC receptors

for itself, and trypsin- and ether-treated IBV, but not for PR8 (Puerto Rican 8) or NDV (Newcastle Disease Virus) (Biswal, 1965; Biswal *et al.*, 1966).

Trypsin-treated IBV reduces the electrophoretic mobility of chicken and turkey erythrocytes, but to a lesser degree than PR8, NDV, or neuraminidase (Biswal, 1963).

The addition of normal or untreated allantoic fluid containing virus eliminates HA by trypsin-treated virus (Nazerian, 1965).

Specific inhibition of HA by both homotypic (Nazerian, 1960) and heterotypic (chicken) (Biswal *et al.*, 1965) has been demonstrated.

Electron Microscopy

Images of biological specimens by transmission electron microscopy (TEM), capable of a magnification to 5 x 10^5, and with an apparent resolution of 0.2 nm, can easily discern morphological characteristics of viruses.

The shadow casting technique (Williams and Wyckoff, 1946) was originally developed to increase the contrast of the image. The oblique deposition, in vacuo, of a thin film of electron-dense material onto the specimens leaves an area on the grid support membrane free of any electron-dense material. Thus, an optically translucent shadow of the image is produced against an opaque background and, by varying the angle and direction of deposition, a three-dimensional image can be constructed.

Negative staining was not successfully applied to electron microscopy until 1954 by Farrant. Later, simpler techniques such as the spray (Brenner and Horne, 1959) and drop (Hurley and Zubay, 1960) were designed for deposition of the specimen onto a plastic film-coated grid. The stain, also serving as a fixative, prevents the effects of dehydration upon the specimen (Johnson and Horne, 1970).

Negatively stained particles are light due to their high translucence (i.e., low scattering power), while the periphery is dark, a result of the high opacity of the metals (i.e., high scattering power). Sub-structural detail, such as capsid symmetry, and the presence or absence of a viral envelope may be elucidated by varying the angle of observation.

Specimens which are appropriately fixed, dehydrated, embedded into plastic, thin-sectioned to no greater than 50 nm in thickness, and stained offer direct observation of specimen internal structure, such as viral cores and nucleic acid orientation.

Virus-erythrocyte interactions have been studied by electron microscopy. However, due to its relative thickness, the erythrocyte must be hemolyzed prior to mixing with virus. Hemolyzed erythrocytes treated with influenza can also be employed for viral morphology without the necessity of viral purification (Dowson and Elford, 1949).

Viral particles observed via negative staining or thin-sectioning have also been isolated from antibody-virus

precipitin bands after immunodiffusion (Watson *et al.*, 1966).

While in TEM an image is developed by primary electrons passing through a thin specimen, scanning electron microscopy (SEM) employs backscattered primary electrons, and secondary electrons radiating from the specimen and hitting a detector, to portray a pseudo, three-dimensional image.

Scanning electron microscopy's current optimal resolution is 10 nm, which limits its application in virology. However, viral budding from host cells (Holmes, 1975) and virus-erythrocyte interactions (Carteaud, 1973) have been successfully observed.

MATERIALS AND METHODS

Virus

Infectious bronchitis virus, 41 (IBV-41), Massachusetts, was used in all experiments.

The virus was cultivated in ten-day-old embryonating chicken eggs, inoculum 0.1 ml per egg, at approximately 10^5 Embryo Infective Doses$_{50}$ (EID_{50}). The allantoic fluid was harvested 48 hours post inoculation, pooled, and either used immediately or stored in 15 ml increments at -72°C.

Erythrocytes

Erythrocytes were obtained by cardiac puncture from Single Comb White Leghorns, using a 2% (w/v) sodium citrate solution as an anticoagulant. The cells were washed by centrifugation (International PR-6) four times, at 1,300 x g for 5 minutes each. The washing agent was either Bacto hemagglutinin buffer (Difco) or a 0.1 M cacodylate buffer at pH 7.2. After the final washing, the supernatant fluid was removed, and the cells were resuspended to a 0.5% (v/v) solution with one of the above buffers.

Trypsin

A 1% (w/v) suspension of trypsin (Difco 1:250) was prepared in double distilled water, incubated for 15

minutes at room temperature, passed through Whatman filter paper, and stored in 2 ml increments at -72°C.

Eggwhite Trypsin Inhibitor

A 1% (w/v) solution of Eggwhite (Ovomucoid) Trypsin Inhibitor (Nutritional Biochemicals Corp.) in double distilled water was prepared and stored in 2 ml increments at -72°C.

Epon-Araldite

A mixture of 20 ml (10.4 g) of araldite 502 (Ladd Corp.), 25 ml (15 g) of Epon 812 (Polysciences), and 60 ml (28 g) of dodecenyl succinic anhydride (Electron Microscopy Science) was prepared, making quite sure that the mixture was thoroughly mixed. The resin was poured in 10 ml increments into screw cap vials and stored at -10°C until used.

Resin was equilibrated at room temperature prior to opening the vial so as not to allow atmospheric moisture to mix with the resin. Then 0.25 ml of DMP-30 (Ladd Corp.) was added to every 10 ml of Epon-araldite to initiate polymerization.

Partial Purification of Virus

Allantoic fluid freshly harvested, chilled, or thawed from -72°C was clarified in an International PR-6 at 2,500 x g for 10 minutes. The supernatant fluid was removed and processed in a Sorval RC-2B ultracentrifuge

with SS-34 rotor, at 17,300-20,200 x g for one hour. The supernatant fluid was removed and the pellet was resuspended in double distilled water, HA buffer, or 0.1 M cacodylate buffer (pH 7.2), appropriate to an experiment.

Trypsinization

A partially purified viral suspension was mixed with 1% trypsin at a ratio of 2:1 and incubated for 1 hour at 56°C, or for 3 hours at 37°C.

After incubation, one part of a 1% solution of trypsin inhibitor was added and the mixture was reincubated for 30 minutes at 23°C to stop the enzymatic reaction.

Negative Staining

All viral preparations, regardless of method, were pre-mixed in various ratios with either a 1-3% solution of phosphotungstic acid (PTA) pH 6.6, a 1% solution of uranyl formate (UF) pH 3.7, a 3% solution of ammonium molybdate (AM) pH 5.2, or several combinations of the above. One drop of the mixture was placed on a 400 mesh EFFA (E. F. Fullam) grid containing a carbon-backed parllodian or formvar film. The excess of the drop was blotted off and the remainder allowed to air dry.

Preparation of Parllodian and Formvar Carbon-Coated Plastic Supports

Clean glass microscope slides were dipped into a beaker containing either a 0.25% (w/v) solution of parllodian in water, or formvar in ethylene dichloride,

and air dried. The edges of the slide were scraped with a razor, lightly blown on, and inserted into a pan of double distilled water at an approximately 35° angle. After the film had detached from the slide it was left floating on the water and allowed to dry further. Carbon grids, 400 mesh EFFA, were then placed on top of the film which was retrieved by placing a metal screen under it and gently lifting the entire film.

After allowing at least 24 hours for the plastic to thoroughly dry, the grids were placed in a Ladd metals evaporator, and a thin carbon coat was applied.

The grids were stored at 4°C.

Hemagglutination from Partially Purified Virus

A partially purified virus suspension from frozen AF (in HA buffer) was prepared, trypsinized, and inhibition of trypsinization accomplished as previously described. From the above mixture, 0.25 ml were mixed with 0.25 ml buffer (HA or 0.1 M cacodylate pH 7.2), plus 0.25 ml of a 0.5% chicken erythrocyte suspension (in HA or 0.1 M cacodylate buffer pH 7.2), gently shaken, and allowed to react for 60 minutes. As a control, identical mixing and incubating schedules were followed; however, trypsin and its inhibitor were replaced with additional buffer (HA or 0.1 M cacodylate pH 7.2).

Thin Sectioning

Thin sections of HA patterns at the bottom of a capillary tube and virus-erythrocyte interactions solidified in ionagar were attempted.

Hemagglutination at Bottom of Capillary Tube

Viral suspensions in cacodylate buffer, trypsinization, inhibition of trypsinization, and controls were all previously described.

Equal volumes of treated or untreated viral suspensions and a 0.5% RBC (in cacodylate buffer) were mixed in a flat-bottomed capillary tube (2 mm x 20 cm) and incubated for 90 minutes. After incubation, the supernatant fluid from agglutinated erythrocytes was removed and equal parts of 4°C stock solutions of 2% glutaraldehyde (0.1 M cacodylate buffer pH 7.2) and 2% osmium tetroxide (0.1 M cacodylate buffer pH 7.2) were added to the reactants and incubated for 2 hours at 4°C.

The fixative mixture was removed and the reactants gently washed three times in the same tube with 0.1 M cacodylate buffer for 10 minutes each. The first two washes were at 4°C, the last at 23°C.

The reactants were then dehydrated in a 10% decimal series of ethanol for five minutes per each increment. Upon removal of the 100% ethanol, propylene oxide was added and the samples were washed three times for 10 minutes each. At the end of the third wash, approximately

one-half of the propylene oxide was removed and replaced with Epon-araldite. The tubes were then placed in a 40°C oven and, as the propylene oxide evaporated, additional Epon-araldite was applied. Approximately 12 hours later the tubes were placed in a 60°C oven to complete polymerization. After 48-72 hours the tubes were broken, the specimens removed and re-embedded in molds containing fresh Epon-araldite. Polymerization schedule was the same as above.

The blocks were sectioned on a LKB-Ultratome III with a Dupont diamond knife. The silver (50-60 nm) sections, picked up with acetone-cleaned 300 and 400 mesh EFFA carbon grids, were stained with 2% uranyl acetate for 30 minutes, rinsed in distilled water, restained for 10 minutes in 0.5% lead citrate containing Triton X-100 (two drops), and rinsed again. Sections were examined on a Phillips EM-300 electron microscope.

Virus-Erythrocyte Interactions in Ionagar

Treated and untreated viral preparations in cacodylate buffer were previously described. One-half milliliter of 1% (w/v) liquid ionagar (37°C) was mixed in a 13 mm x 75 mm glass tube with 0.25 ml of trypsin-treated and untreated viral suspension, and 0.25 ml of 0.5% RBC (cacodylate buffer), and allowed to incubate for 75 minutes at 37°C. Upon completion of incubation, the mixture was brought to room temperature for 30 minutes to assure solidification.

The tubes were broken, gels removed, and sliced into cubes of not more than 1 mm.

The cubes with the greatest density of RBC were placed into screw cap vials containing 0.1 M cacodylate buffer at 4°C.

Equal parts of 4°C stock solutions of 2% glutaraldehyde (cacodylate buffer pH 7.2) and 2% osmium tetroxide (cacodylate buffer pH 7.2) were mixed, the buffer removed, the fixatives added, and the mixture incubated 60 minutes at 4°C. The fixatives were then removed, and the specimens washed twice with 0.1 M cacodylate buffer for 20 minutes per wash.

Dehydration was by an ethanol series of 10% decimal series increments for a period of five minutes each, followed by two additional washes in propylene oxide of five minutes each.

At the end of the second wash, the propylene oxide was removed and a 2:1 mixture of propylene oxide to Epon-araldite was added for 30 minutes. An additional three parts Epon-araldite were added for 60 minutes, followed by replacement with 100% Epon-araldite.

The cubes remained in 100% Epon-araldite overnight, were then removed and placed in molds containing fresh resin. The molds were placed in a 40°C oven for 18 hours, then transferred to a 60°C oven for 48-72 hours to complete polymerization.

The blocks were trimmed, thin sectioned, stained, and examined as previously described (HA in bottom of capillary tube).

RESULTS

Effects of Temperature and Centrifugation on Viral Particles

Allantoic fluid (AF) of infected chicken embryos serves as the medium in which the virus is commonly isolated. To enhance virion appearance, treatment of AF to various conditions and procedures was considered (Table 1).

Freshly harvested AF, without further treatment, was unsatisfactory in electron microscopy due to extraneous particulate matter masking or distorting the virions.

Table 1. Variables considered in preparation of allantoic fluid

Status of AF	Temperature (°C)
fresh	23
clarified	23
pelleted	23
fresh	4
clarified	4
freeze-thaw	-72 - 23

All specimens were negatively stained with 1% PTA (pH 6.6) by the drop method, onto carbon-backed, 400 mesh formvar grids.

There were few particles with viral features in the clarified AF at each temperature, again due to masking. The pellet, resuspended in double distilled water, from previously clarified AF, contained numerous, easily observable particles. The tulip-like projections (Figure 1) were readily seen and there was little obstruction from extraneous material.

Optimum results were obtained with the freeze-thaw technique, whereby freshly harvested AF pools were stored at -72°C for several days, thawed at 23°C in a water bath, clarified, pelleted, and resuspended as previously described. Viral particles were abundant (Figure 2), with good resolution of projections (Figure 3), and minimum obstruction by extraneous macromolecules.

From the above observations, the following inferences may be made: 1) temperature changes produce little, if any, drastic effects on viral structure; thus viruses may be subjected to severe temperature shifts (e.g., freeze-thaw) and still maintain their structural integrity; 2) harsh treatment by ultracentrifugation has no apparent adverse effects on viral surface projections; 3) the number of viral particles per given grid area is increased by pelleting and resuspending in a smaller (relative to AF) volume; 4) the freeze-thawing of AF removes further extraneous material, permitting less obstruction of viral particles.

Figure 1. Pelleted material from freshly harvested AF. Magnification 512,500 X.

Figure 2. Allantoic fluid was frozen, thawed, clarified, then pelleted. Magnification 53,000 X.

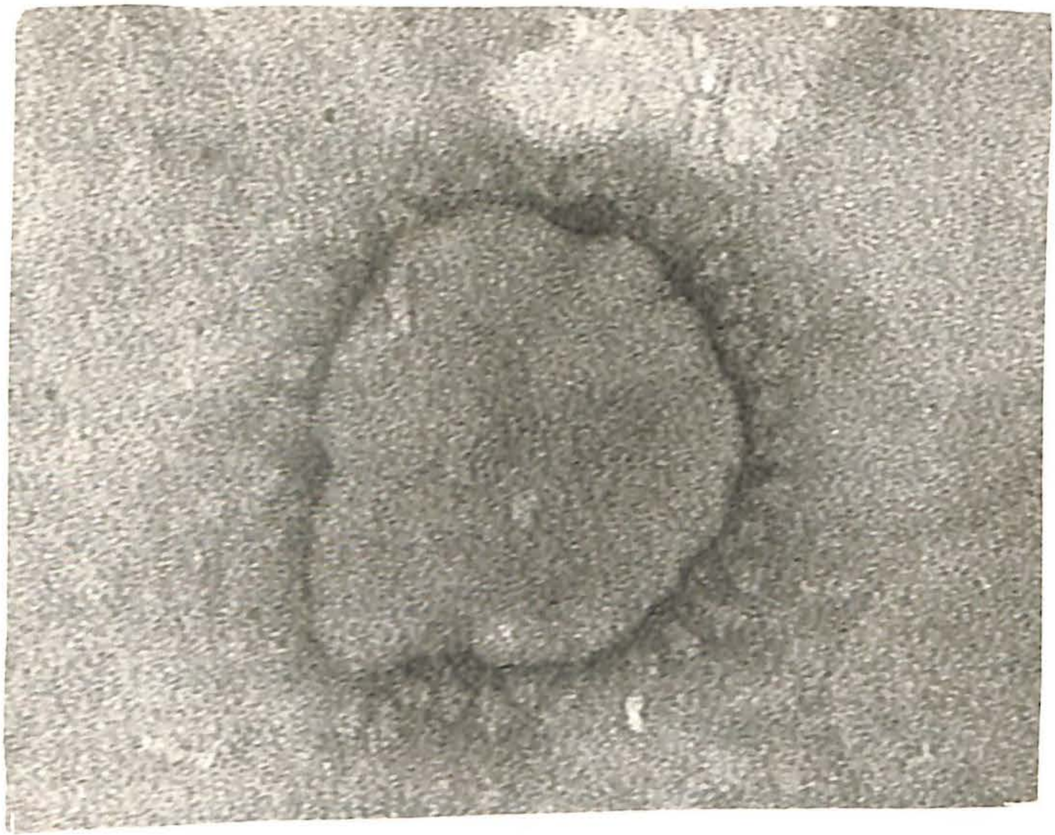

Figure 1

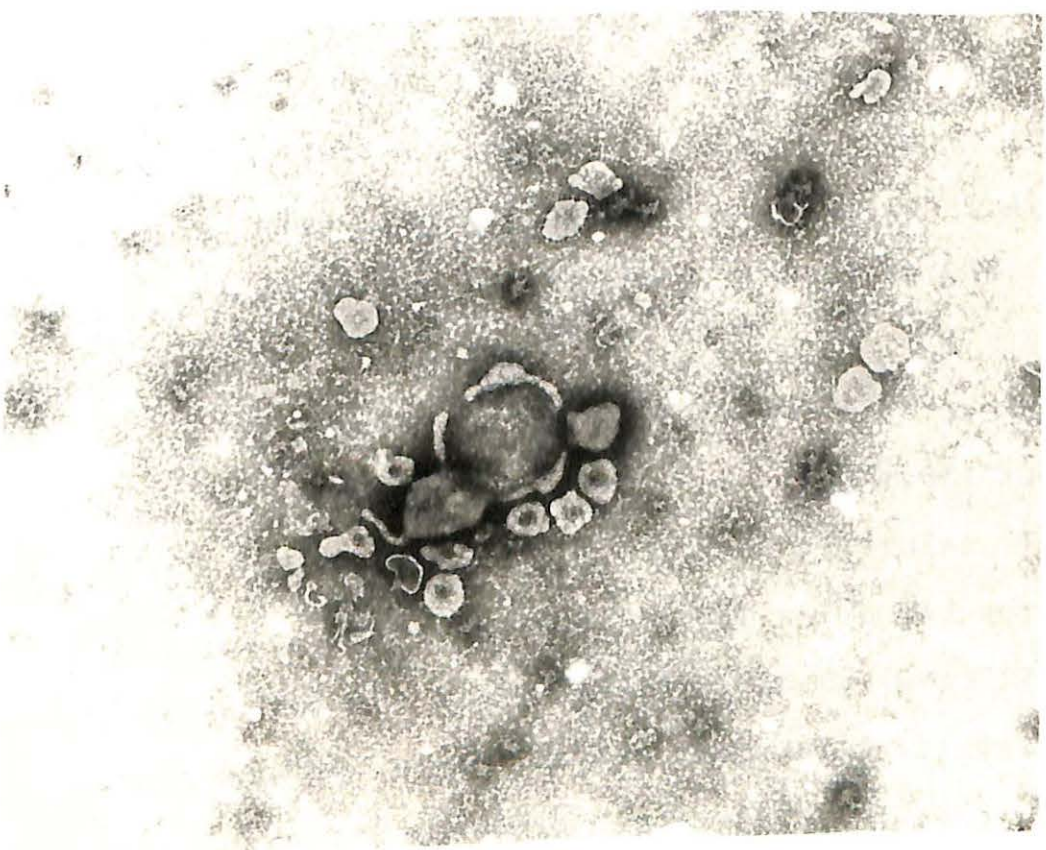

Figure 2

Effects of Glutaraldehyde upon Viral Particles

Attempts to maintain the natural configuration of the virus, and to prevent possible shearing of surface projections during centrifugation, were examined using fresh AF fixed with various concentrations of glutaraldehyde (cacodylate buffer, pH 7.2), conditions of temperature, time, status of fluid when fixed, and procedure.

To determine during which stage of virus preparation the AF should be fixed, fresh, clarified, and resuspended (from pellet) portions were mixed with glutaraldehyde (Table 2). Microscopy of freshly harvested AF was the same as without fixation, in that there was too much extraneous material background for differentiation of virions. Fixed, clarified fluid, at all temperatures, was clear and suitable for microscopy. Projections were distinct, plentiful, and suffered very little obstruction (Figure 4). Pellets resuspended after one hour at 107,240 x g contained high populations of viral particles, without, however, comparable clarity to those present in clarified AF (Figure 5).

To examine virions in their "natural" state, clarified AF was fixed during various stages of preparation at host (37.7°C) and near freezing (4°C) temperatures (Table 3). Best results were obtained from AF which had not been frozen. Clarified AF, fixed for five minutes at 37.7°C, then immediately pelleted, yielded numerous particles, little obstruction by extraneous material, and clear

Table 2. Consideration of various stages of fixation at different temperatures and percentage fixative

Status of AF	Temperature during fixation (°C)	Percent Glutaraldehyde
fresh	23	1.0
clarified	23	1.0
pelleted	23	2.0
fresh	23	0.5
clarified	23	0.5
pelleted	23	0.5
fresh	4	1.0
clarified	4	1.0
pelleted	4	2.0
fresh	4	0.5
clarified	4	0.5
pelleted	4	0.5

All specimens were negatively stained with 1% PTA (pH 6.6) by the drop method, onto carbon-backed, 400 mesh parllodian grids. Length of fixation was the time necessary for clarification (10 minutes), or pelleting (60 minutes).

Figure 3. Allantoic fluid was frozen, thawed, clarified, then pelleted. Magnification 400,000 X.

Figure 4. Clarified allantoic fluid previously fixed with 1% glutaraldehyde at 23°C. Magnification 200,000 X.

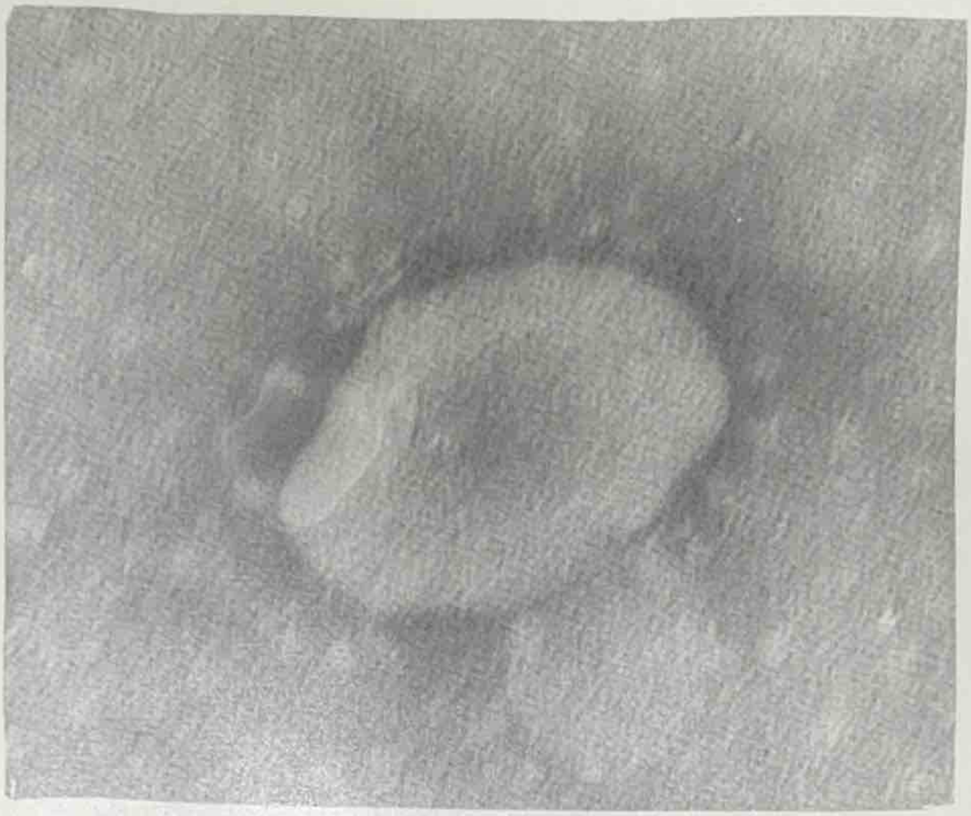

Figure 3

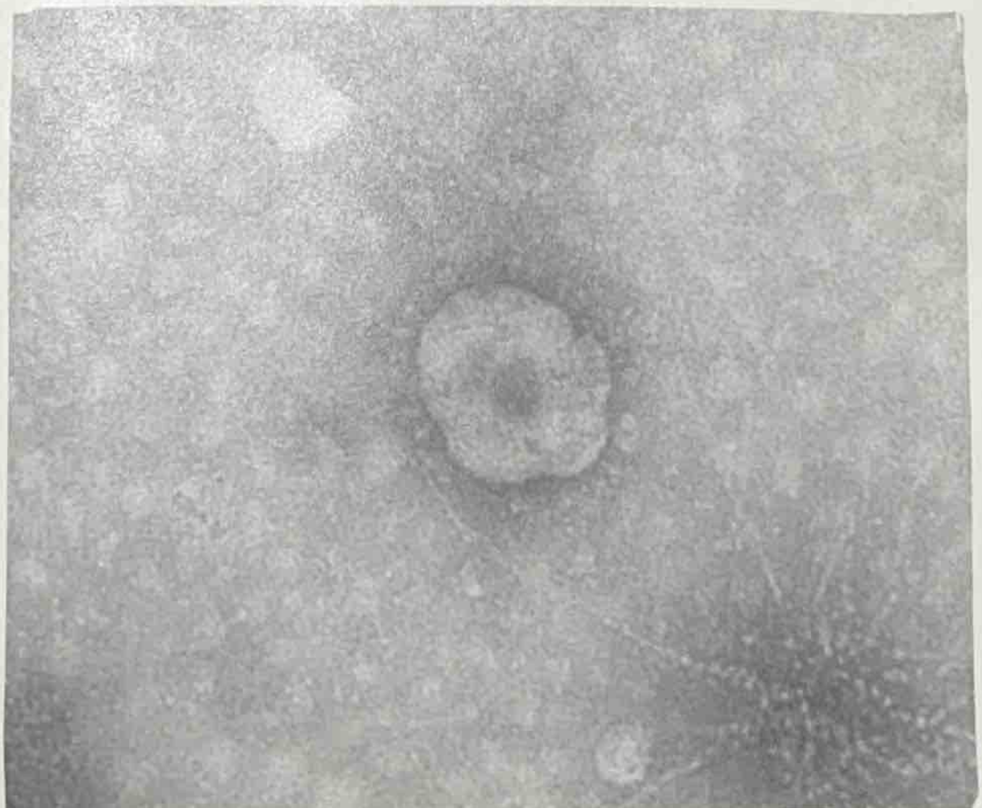

Figure 4

Figure 5. Pelleted material, fixed prior to clarification with 1% glutaraldehyde at 4°C. Magnification 512,500 X.

Figure 6. Specimen fixed, prior to pelleting, with 1% glutaraldehyde for 5 minutes, at 37.7°C. Magnification 257,500 X.

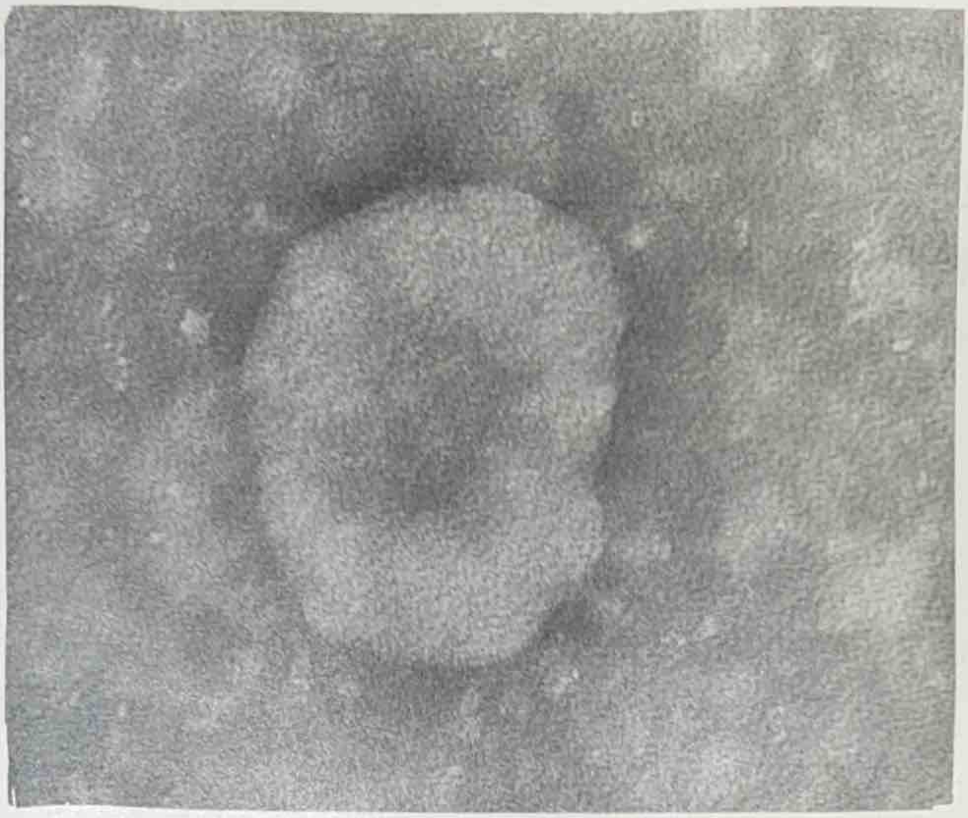

Figure 5

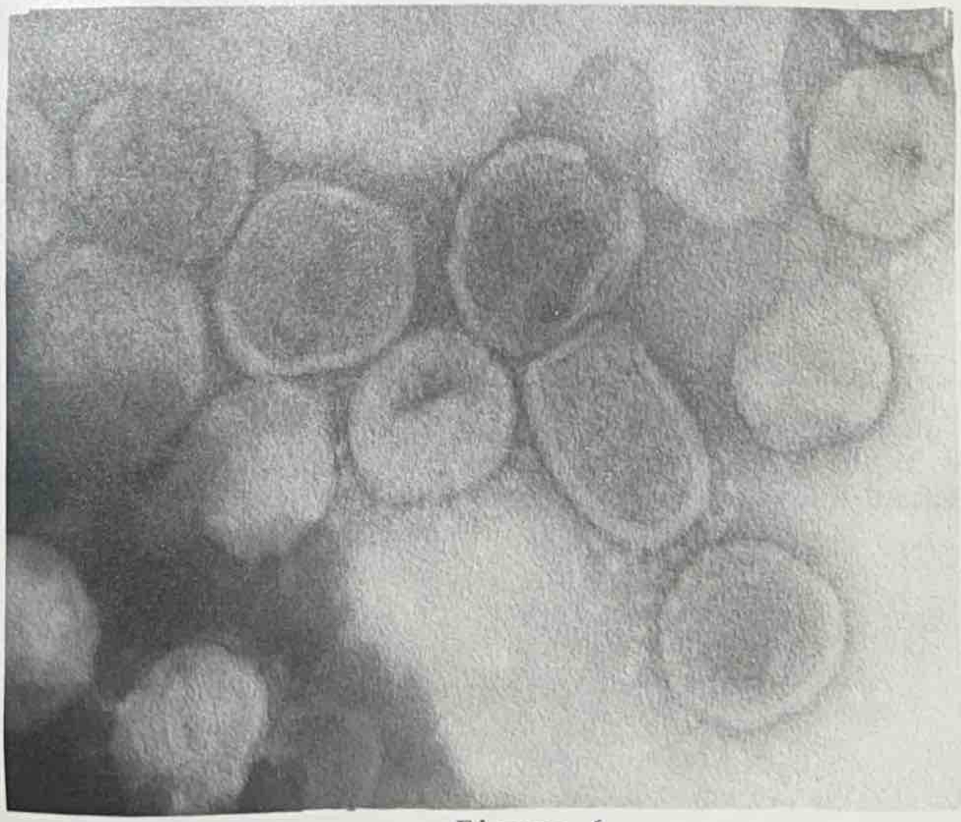

Figure 6

Table 3. Consideration of different fixation schedules of clarified allantoic fluid at 37.7°C or 4°C for various lengths of time

Schedule	Fixation time (minutes)	Temperature during fixation (°C)
clarify-fix-pellet	5	37.7
"	15	37.7
"	30	37.7
"	5	4
"	15	4
"	30	4
clarify-freeze-thaw-fix-pellet	5	37.7
"	15	37.7
"	30	37.7
"	5	4
"	15	4
"	30	4
clarify-fix-freeze-thaw-clarify-pellet	5	37.7
"	15	37.7
"	30	37.7
"	5	4
"	15	4
"	30	4

All specimens were fixed in 2% glutaraldehyde (cacodylate buffer, pH 7.2), and negatively stained with 3% PTA (pH 6.0) by the drop method, onto carbon-backed, 400 mesh parllodian grids.

projections (Figure 6). Viral particles from fluid fixed prior to freezing had distorted peripheries, particularly near surface projections (Figure 7). Fluid fixed after freeze-thawing offered little advantage either.

Virions mildly fixed possess many surface projections which are clearly distinguishable, and in most instances are superior to unfixed material. The majority of surface projections are distorted, however (Figure 8), due to inter-protein linkages between extraneous material and virions by glutaraldehyde. Unfixed material is technically simpler to prepare, and provides more virions with distinct projections.

Staining and Application

Experiments to enhance peripheries and projections of viral particles were performed with various stains possessing inherent differences in molecular weight, protein attraction, and spreading ability (Table 4).

The metallic salts in 1% PTA penetrated well between the tightly packed surface projections, and produced a high-contrasting background (Figure 4). Three percent PTA, while producing a comparable background, obscured the top of the projections due to the excessive stain (Figure 9).

Ammonium molybdate (3%, pH 5.2) spreads the most uniformly, penetrates well between projections, highlights membranes well, but lacks the dark background of PTA, thus providing, in most instances, poorer contrast and resolution (Figure 10).

Figure 7. Clarified material, previously fixed with 1% glutaraldehyde for 30 minutes, at 4°C, was thawed, clarified further, then pelleted. Magnification 312,500 X.

Figure 8. Example of distorted surface projections due to glutaraldehyde. Magnification 200,000 X.

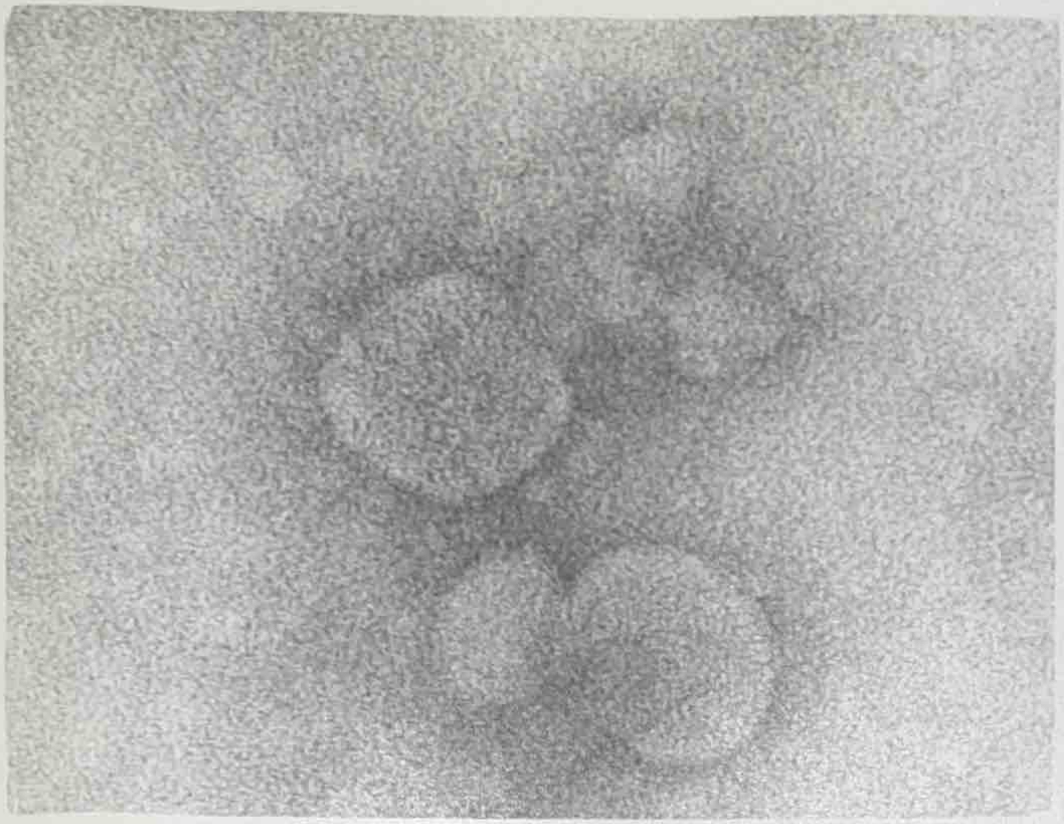

Figure 7

Figure 8

Figure 9. Viral particle stained with 3% PTA (pH 6.0). Magnification 200,000 X.

Figure 10. Viral particle stained with 3% AM (pH 5.2). Magnification 257,500 X.

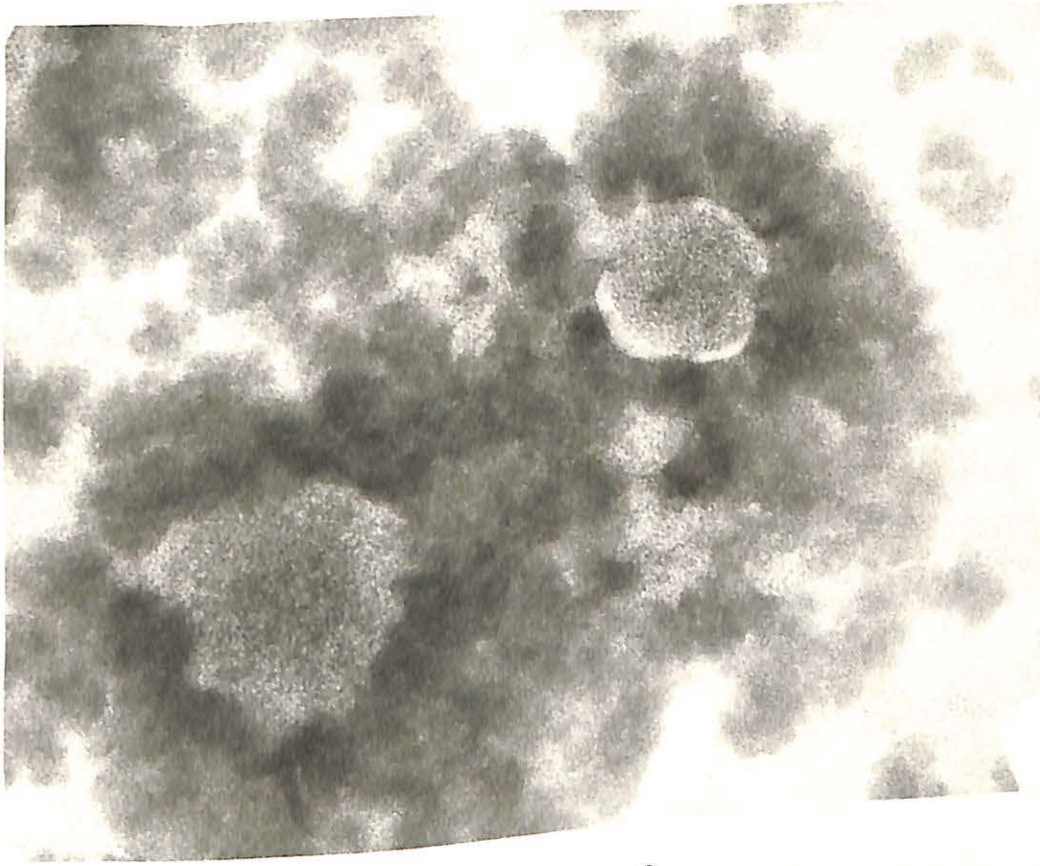

Figure 9

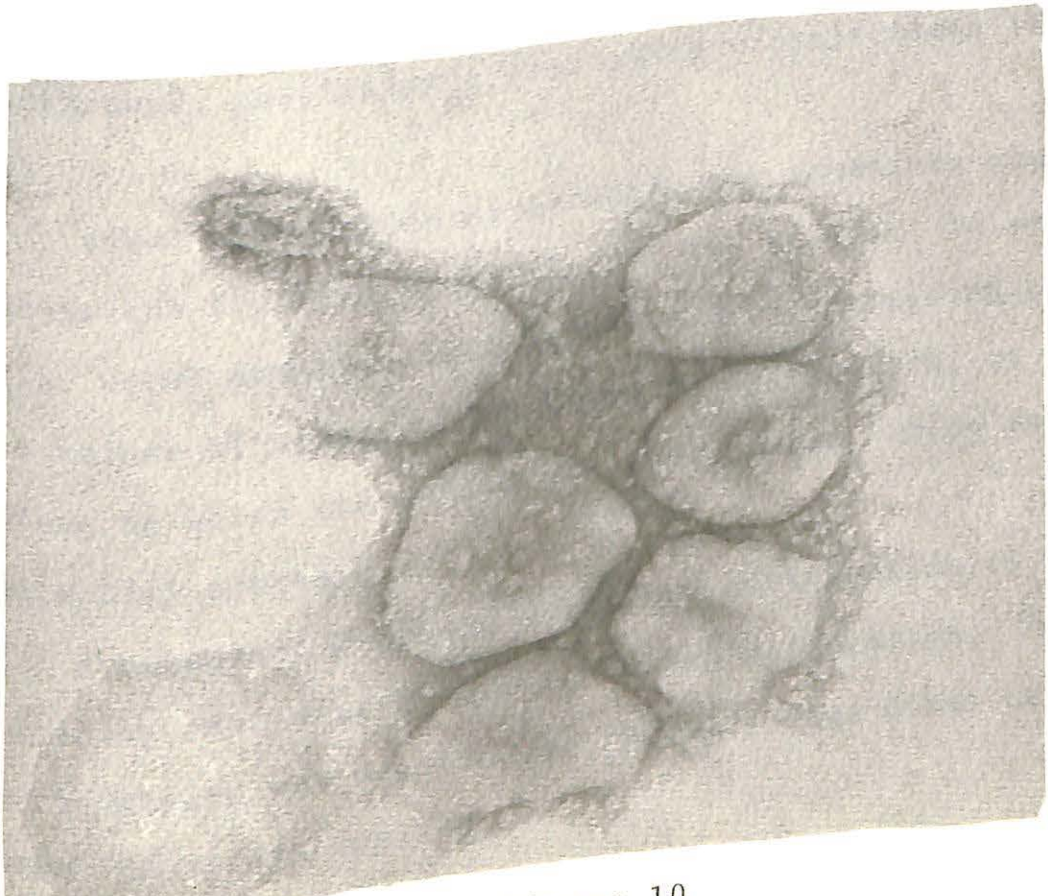

Figure 10

Table 4. Various stains used to highlight virions

Stain	Percentage	pH
PTA	1	6.6
PTA	3	6.0
UF	1	3.7
AM	3	5.2

All specimens examined were from freshly harvested AF, clarified, fixed with 0.5% glutaraldehyde for 5 minutes, at 37°C, pelleted, and resuspended in 1:1 double distilled water.

Uranyl formate (1%, pH 3.7), by virtue of its low molecular weight, is able to penetrate well between projections, but provides poor contrast, and a granular background, particularly near surface projections (Figure 11).

Viral aggregation or crowding may sometimes occur due to the fixative properties of the stains. To resolve this, viral density could be reduced by increasing either the volume of stain, which added to aggregation, or the volume in which the pellet was resuspended, which was the successful approach.

A nebulizer produced a uniform distribution of viral particles onto a grid, and prevented aggregation. However, viral density was drastically reduced, regardless of the number of sprays applied.

Figure 11. Virion stained with 1% UF (pH 3.7). Magnification 200,000 X.

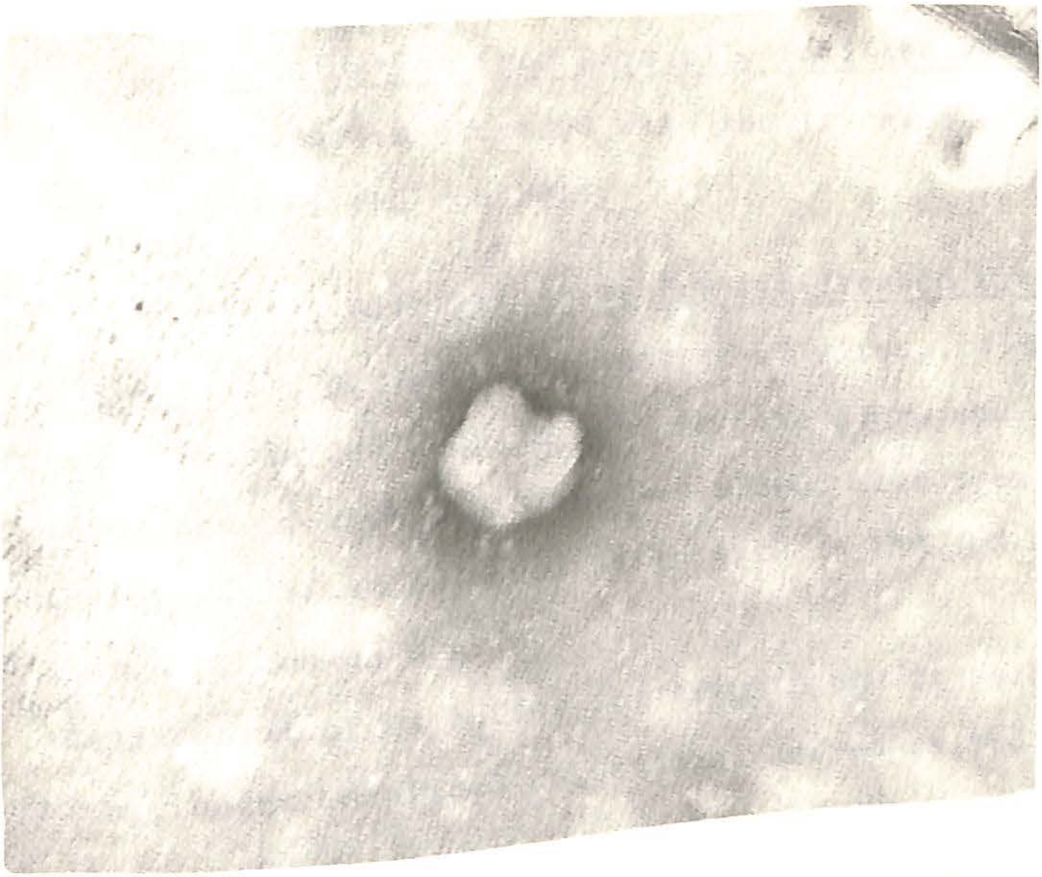

Figure 11

Hemagglutination from Partially Purified Virus

Hemagglutination occurred with the trypsinized sample, but not in any of the controls.

Structural Alterations of Trypsin-Treated Virus

A partially purified preparation of virus, in HA buffer, was trypsinized both at 56°C for one hour, and at 37°C for three hours, then inhibitor was added. The treated suspension was mixed in equal parts with 1% PTA (pH 6.6) and a drop placed on a grid.

Replacement of HA buffer for trypsin and trypsin inhibitor, under identical conditions, served as the control.

Two distinct types of viral particles were observed in both treated and nontreated samples: a "normal" virion, representative of those most commonly seen, and a "dark center" virion, with an opaque center and translucent periphery (Figure 12). The treated suspensions, at 56°C, contained more "dark center" virions, whereas the control contained more of the "normal" virions (Figure 14).

To determine whether adverse temperatures (i.e., 56°C), rather than trypsinization, produced the appearance of these "dark centers", viral suspensions were treated with trypsin (or additional HA buffer) at 37°C for three hours. There was a greater percentage of "dark centers" in the treated sample than either the control at 37°C, or the treated

Figure 12. Example of both types of virions characterized. Magnification 270,000 X.

Figure 13. Example of "suspected" virion. Magnification 507,500 X.

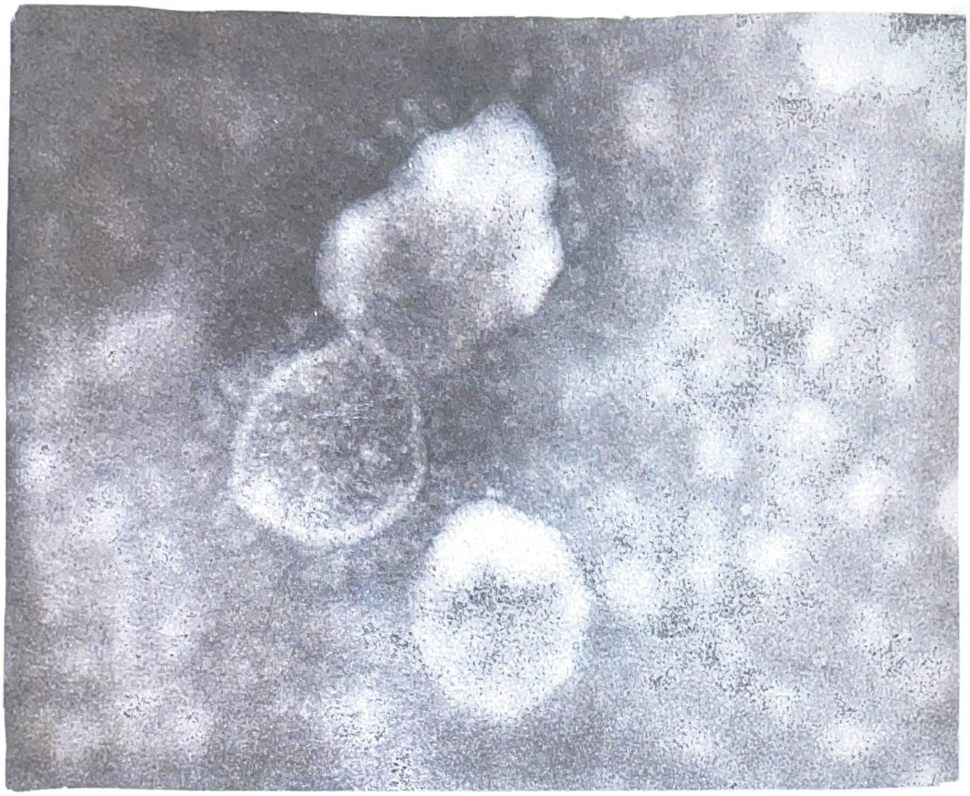

Figure 12

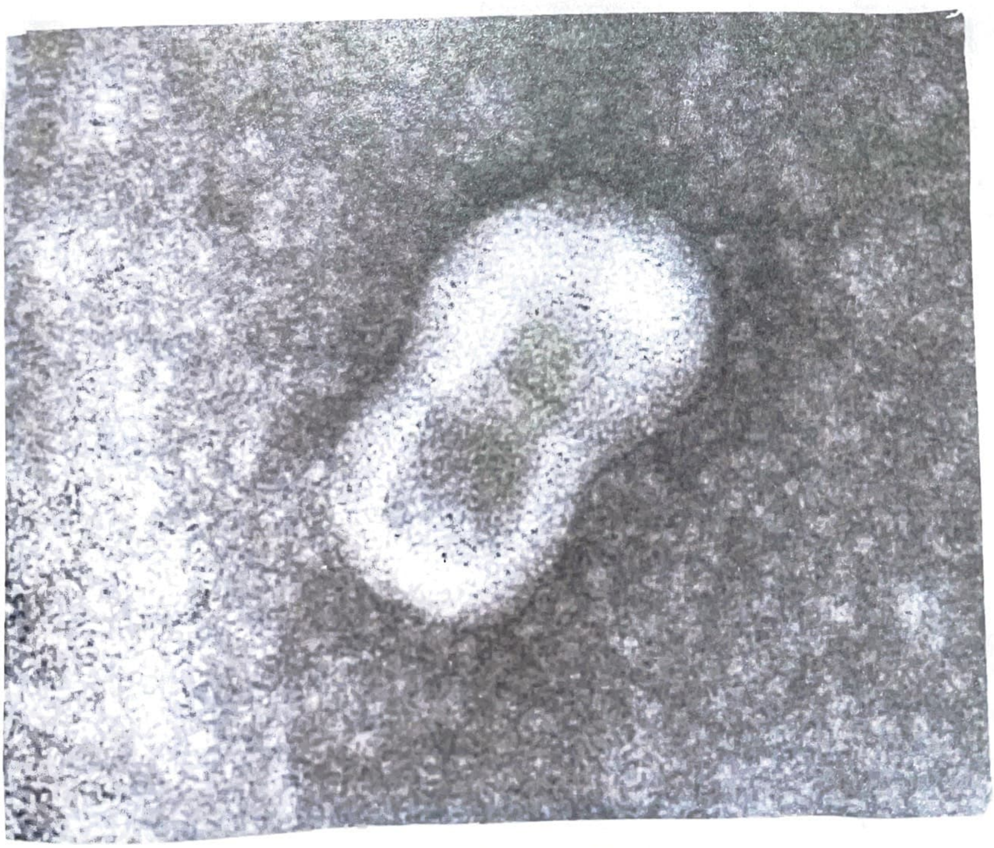

Figure 13

Figure 14. Assay 1: dark center versus normal virions.

a) Control at 23°C (examined directly from partially purified preparations).

b) Trypsin-treated and incubated for 1 hour at 56°C.

c) Nontreated and incubated for 1 hour at 56°C.

d) Trypsin-treated and incubated for 3 hours at 37°C.

e) Nontreated and incubated for 3 hours at 37°C.

triangles = normal

squares = dark center

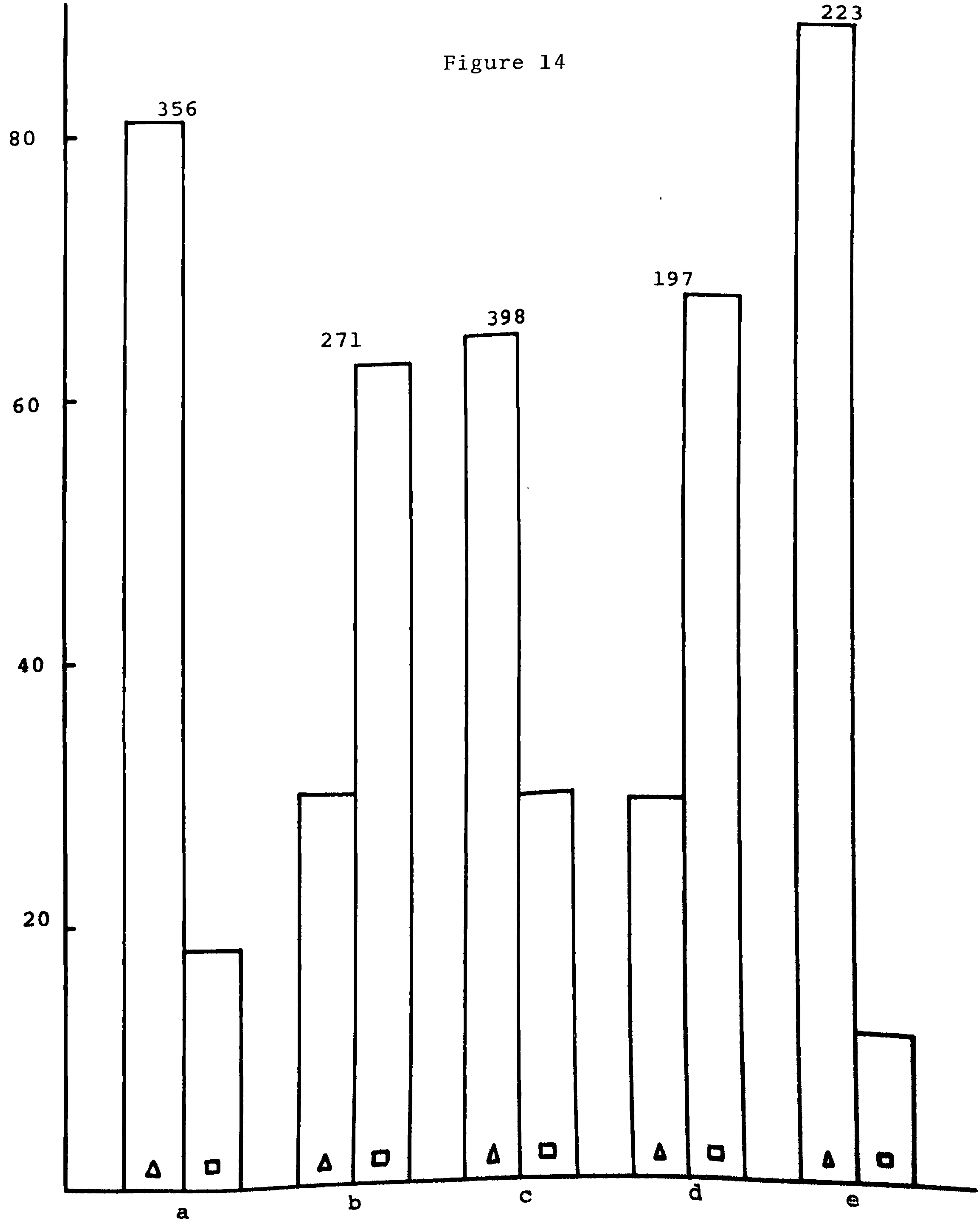
Figure 14
80
60
40
20
356
271
398
197
223
a
b
c
d
e

sample at 56°C. In addition, the percentage of "normal" particles in the 37°C control was greater than in the 23°C control, or in the 56°C control (Figure 14).

Both treated and nontreated samples contained "suspected dark center" virus-like particles (Figure 13) which, although containing an opaque center and translucent periphery, lacked projections. Fewer "suspected" than "confirmed" (i.e., containing surface projections) were observed in any of the treated or control preparations. More "suspected" particles were seen in treated than nontreated samples, and samples treated at 56°C contained more than samples treated at 37°C (Figure 15). Considering "suspected dark center" virions to be "confirmed" virions (Figure 16), the percentage differences between them, for either assay, are quite similar (Figure 14 versus Figure 16).

To determine other possible structural differences, samples were also stained in an ethanol series of 25, 50, 75, 95, 100%, containing 0.5% uranyl acetate. While relatively more "dark center" virions were seen in the control samples, percentage differences between "dark center" and "normal" virions were similar.

Figure 15. Assay 2: normal versus "confirmed" and "suspected" dark center viral particles.

a) Control at 23°C (examined directly from partially purified preparations).

b) Trypsin-treated and incubated for 1 hour at 56°C.

c) Nontreated and incubated for 1 hour at 56°C.

d) Trypsin-treated and incubated for 3 hours at 37°C.

e) Nontreated and incubated for 3 hours at 37°C.

triangles = normal

squares = confirmed

circles = suspected

Figure 15

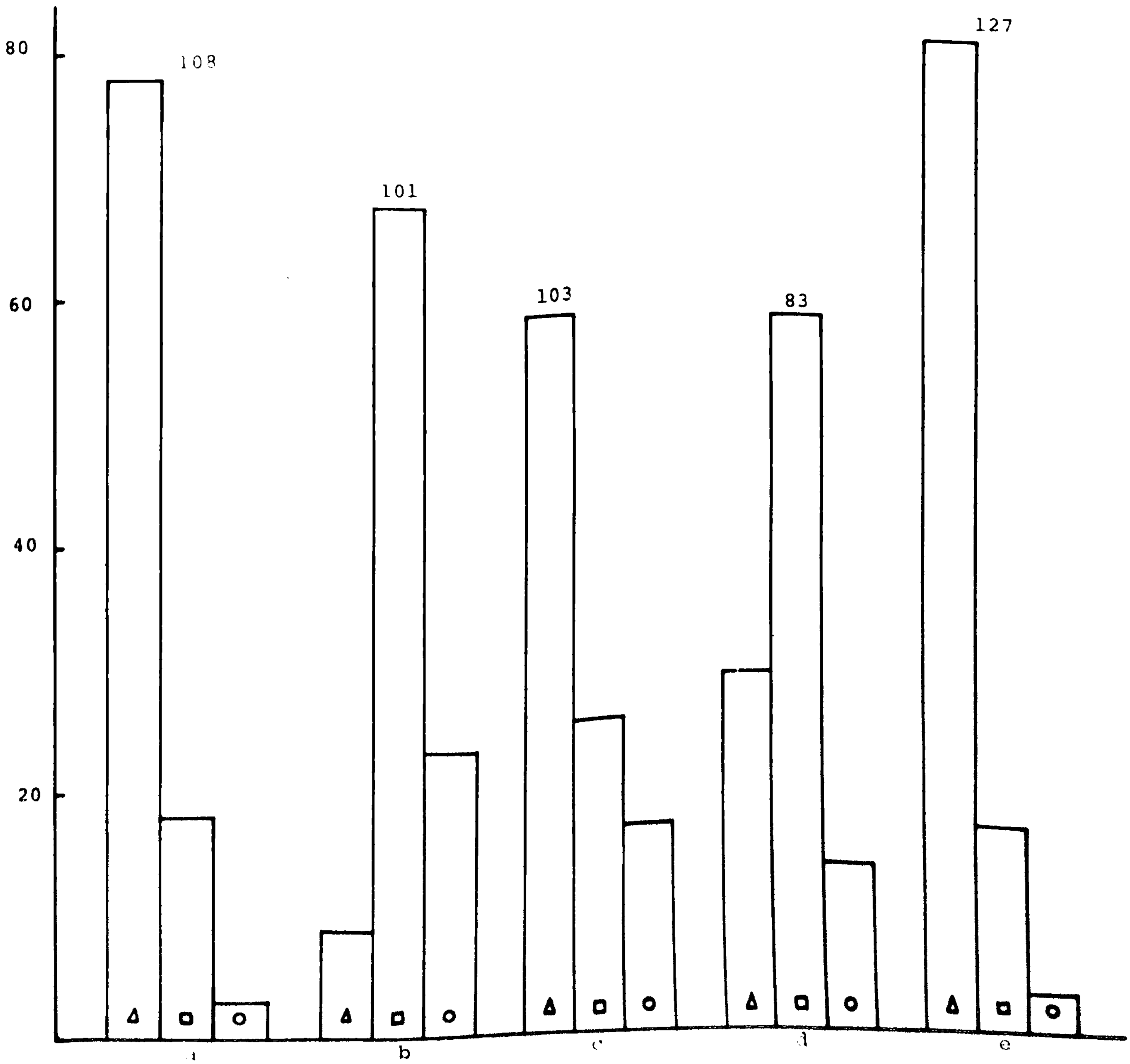

Figure 16. Dark center versus normal, where dark dark centers are considered as both "suspected" and "confirmed" viral particles.

a) Control at 23°C (examined directly from partially purified preparations).

b) Trypsin-treated and incubated for 1 hour at 56°C.

c) Nontreated and incubated for 1 hour at 56°C.

d) Trypsin-treated and incubated for 3 hours at 37°C.

e) Nontreated and incubated for 3 hours at 37°C.

triangles = normal

squares = dark center

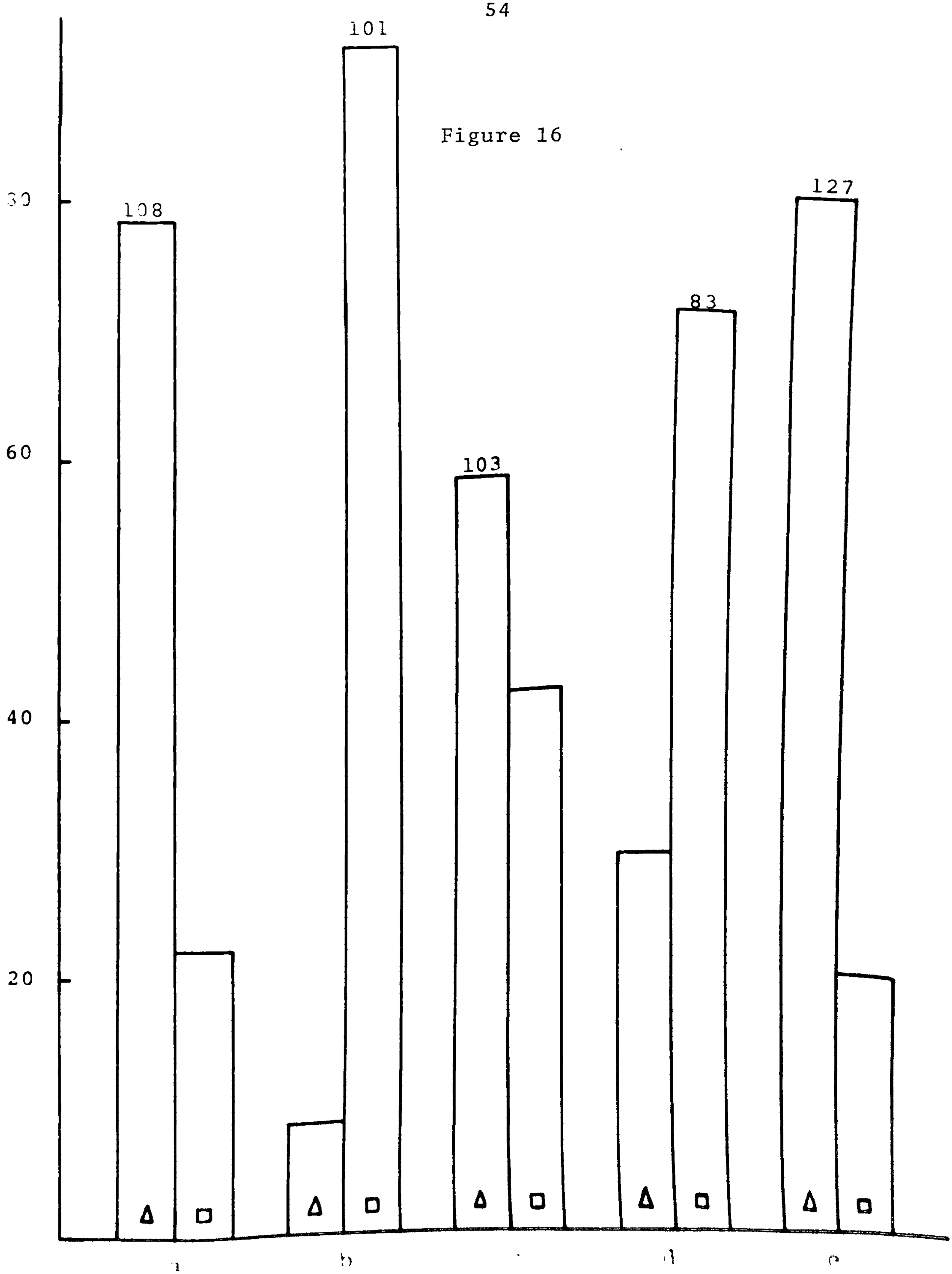

Figure 16

Hemagglutination

Capillary Tube

The capillary tube replaced the standard HA tube to provide a greater density of virions and RBC suitable for thin sectioning.

Virus-like particles were associated with erythrocytes (Figure 17) in both treated and nontreated samples. In the latter, the virions might have been entrapped by descending RBC, then later non-specifically attached by glutaraldehyde. More than twice as many virus-like particles were associated with RBC in the treated than in the nontreated samples (6.4 particles/RBC versus 2.7 particles/RBC, respectively), the difference possibly being a specific virus-erythrocyte reaction, as opposed to non-specific attachment by glutaraldehyde. The average number of virus-like particles on the surface of one RBC could be exceedingly higher if considered on a three-dimensional rather than two-dimensional (50 nm thick section) basis. The ratio, however, should remain constant.

Morphological differences between treated (Figure 18) and nontreated (Figure 19) virus-like particles were difficult to discern in thin sections. Nontreated particles appeared to be more intact and less disrupted than treated particles. Projections were not evident in either sample.

Figure 17. Virus-like particles associated with an erythrocyte. Magnification 81,000 X.

Figure 18. Trypsin-treated virus-like particle associated with an erythrocyte. Magnification 437,500 X.

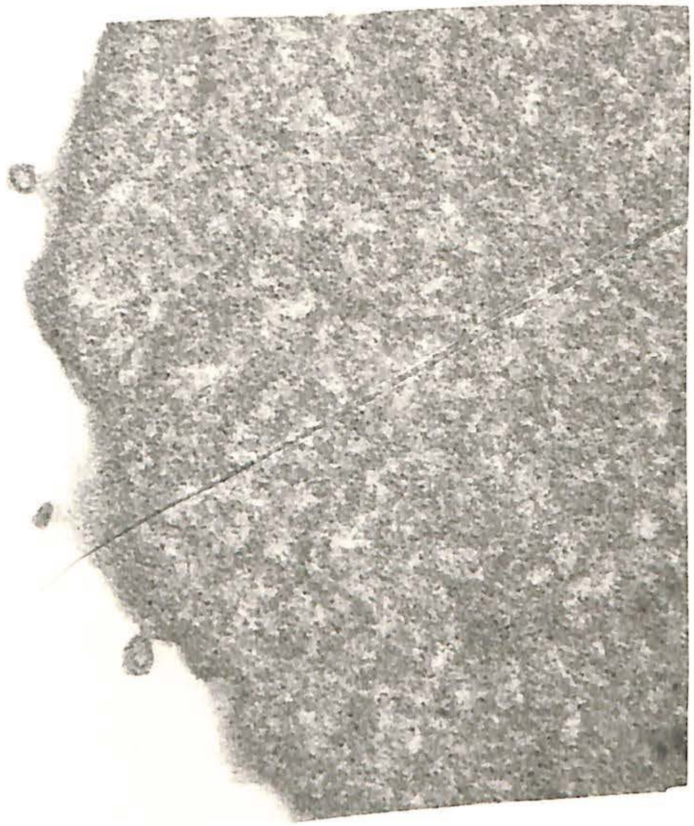

Figure 17

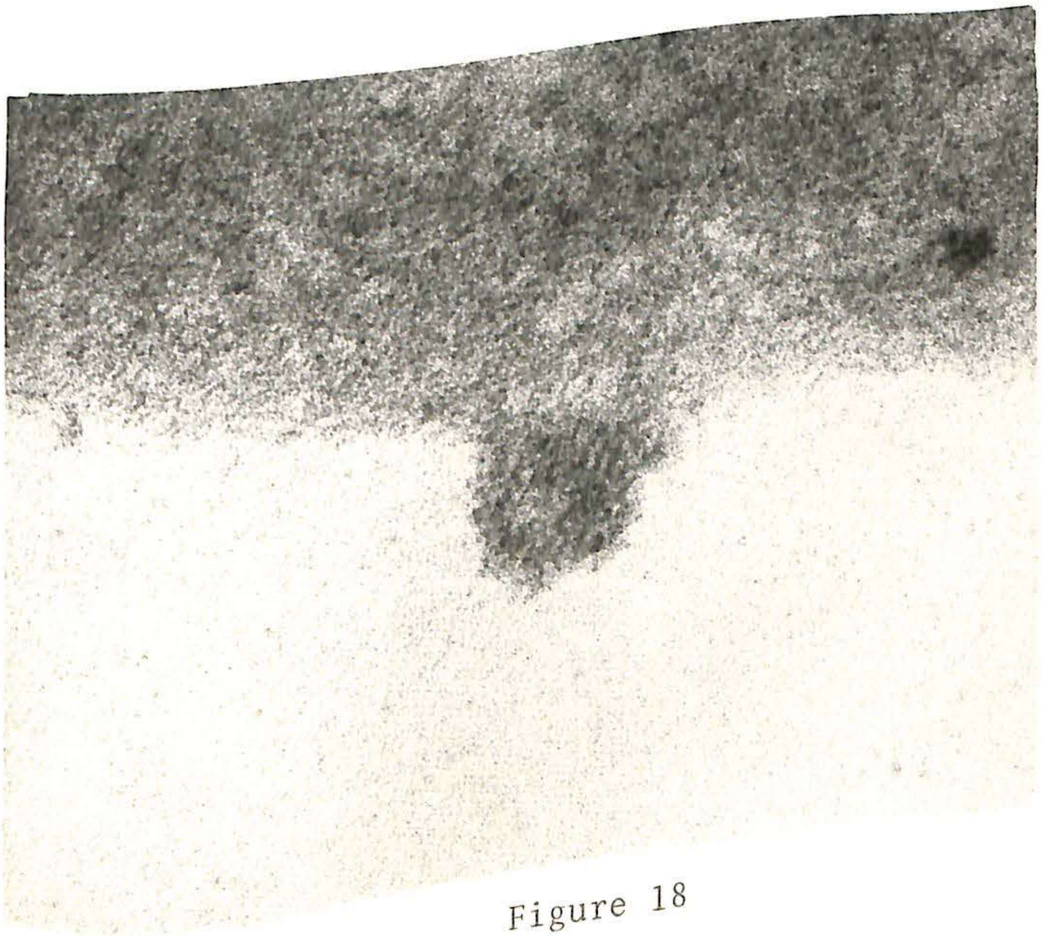

Figure 18

Figure 19. Nontreated virus-like particle. Magnification 347,500 X.

Figure 20. Virus-erythrocyte suspensions in ionagar after 75 minutes incubation.

Figure 19

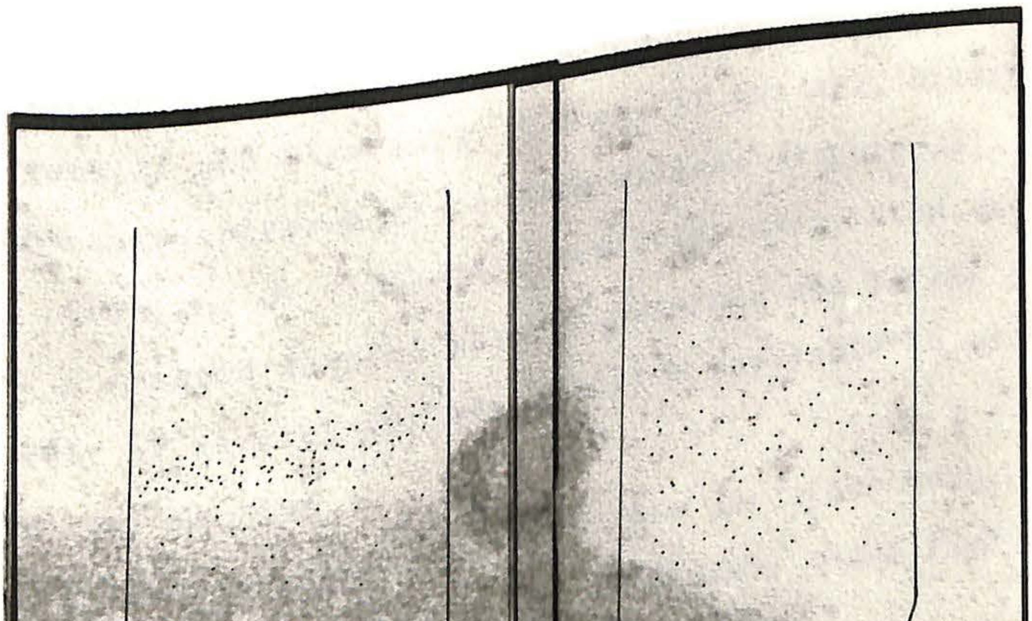

Figure 20

Ionagar

To avoid non-specific attachment of virions to RBC, possible virus-erythrocyte interactions were immobilized when the ionagar solidified.

The mixture containing treated viral suspensions formed a stratum of RBC in the middle of the solution, prior to, and after, solidification of the ionagar, while the nontreated suspension contained a dispersed, homogeneous population of RBC (Figure 20).

Virus-like particles associated with erythrocytes were only observed in the treated sample. Although particles were observed in the nontreated sample, none were associated with RBC.

Bridging of adjacent erythrocytes by virus-like particles was observed in treated viral suspensions (Figure 21 and Figure 22). Some, if not all, bridge components appeared to contain virions (Figure 23).

Counting of virus-like particles associated with RBC in treated samples was not possible due to the low density of viruses and erythrocytes available in any one given gel section.

Neither bridges nor virus-like particles were observed in suspensions of erythrocytes, under identical conditions, when solidified in ionagar.

Figure 21. Cluster of virion-like particles associated with erythrocyte. Magnification 507,500 X.

Figure 22. Bridge annexing two adjacent erythrocytes. Magnification 507,500 X.

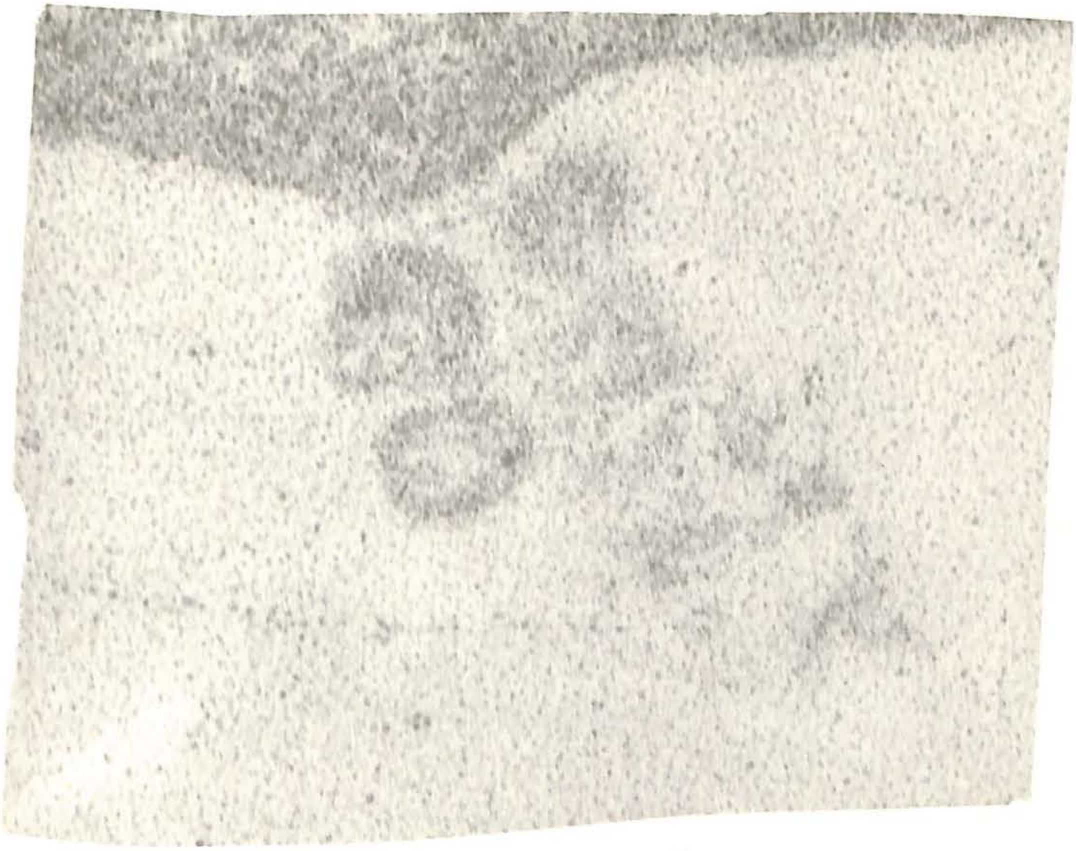

Figure 21

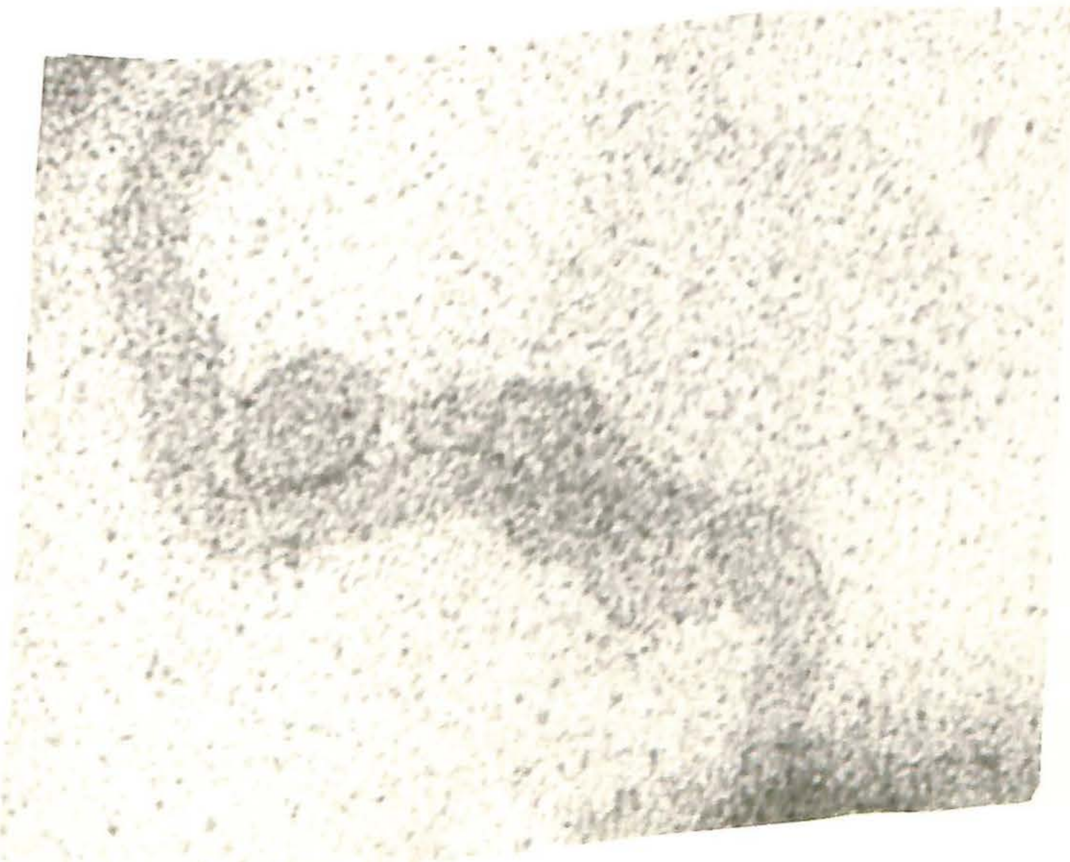

Figure 22

Figure 23. Bridge components. Magnification 641,250 X.

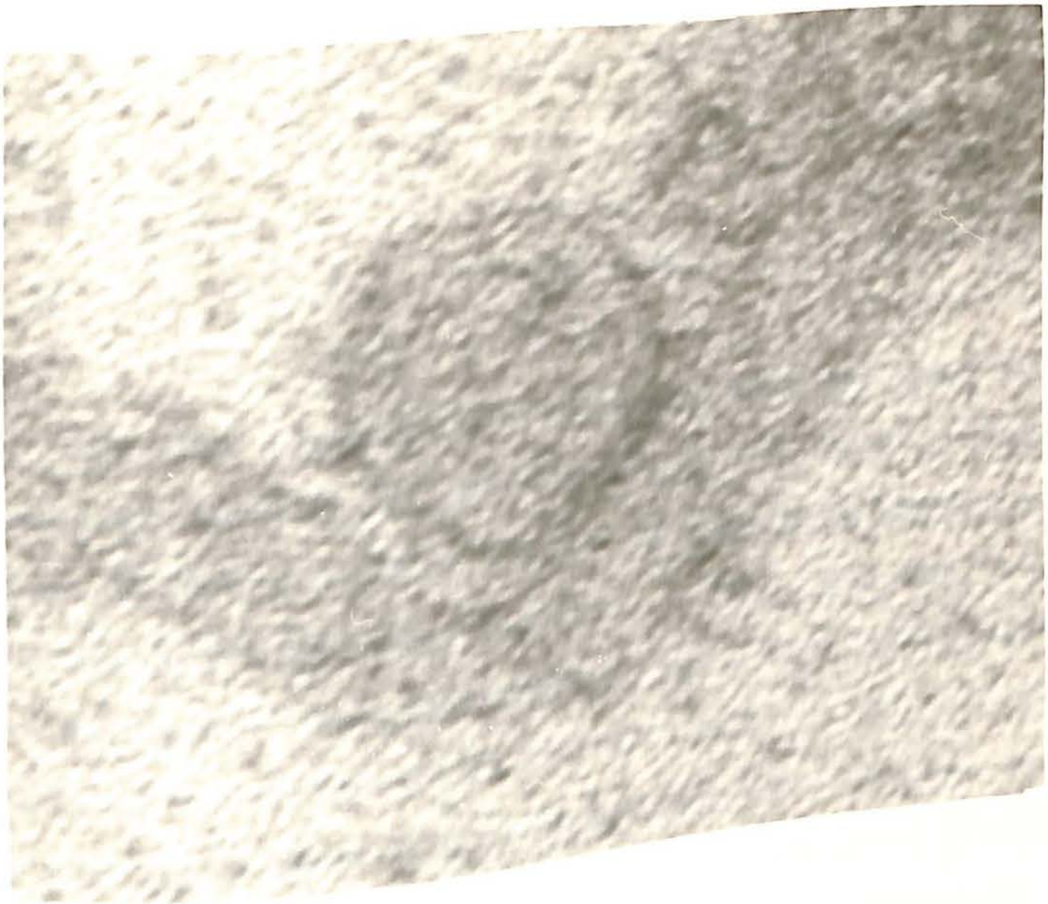

Figure 23

DISCUSSION

Previous electron microscopy of IBV isolated from crude allantoic fluid (Marsolias *et al.*, 1971), pelleted crude AF (Berry *et al.*, 1964), and pelleted material previously frozen, thawed and clarified (Almeida, personal communication), has provided the potential of examining the virus by a variety of techniques.

In the present study, particles were evident under all conditions of temperature and preparation, except directly from crude AF with its extraneous material. The freeze-thaw technique denatures and increases aggregation of proteins. This technique, modified from Almeida and Waterson (1969), increased separation of viral particles from extraneous AF after clarification, and is a gentle means for partially purifying viral suspensions.

Previous investigators (Harkness and Bracewell, 1974) have commented on the potential fragility of IBV surface projections. Such fragility was not encountered in this laboratory. Virions subjected to various stresses maintained their structural integrity. Such discrepancies may be due to differences in either the number of egg-passages of the isolates or specimen preparation, prior to examination.

Partially purified viral preparations were used to examine the effects of trypsin on IBV, and the subsequent virus-erythrocyte interactions. So far as is known, this is the first study in which partially purified preparations were used for this reaction. Previous studies were conducted with crude AF viral preparations. Although the objectives have been the same in most instances, it must be emphasized that differences in preparations may lead to different results.

The effects of trypsin on IBV has been subject to great debate. Nazerian (1960) suggested that virions were reduced in size by the removal of the envelope by trypsin. In contrast, Berry *et al.* (1964), examining nonclarified, pelleted material, stated that trypsin did not structurally alter the virion.

Data in this present study indicate a greater propensity of "dark center" particles in the treated sample, which suggests that trypsin does produce some structural changes. The "dark centers" may be interpreted as a weakening in the membrane of the virus enabling more stain to accumulate in a concavity, the loss of part of the membrane, replaced by stain, or the release of ribonucleoprotein material. Each explanation would account for decreases in virion size.

Berry's group used pelleted crude AF, thus concentrating extraneous protein material in addition to the viral particles. Considering that trypsin is capable of

digesting only a constant amount of protein substrate per unit time, the probability of the enzyme affecting IBV is drastically reduced due to the greater amount of available protein. Thus, the particles may not have been exposed to trypsin prior to examination. In addition, the Massachusetts strain (IBV-41) capable of HA activity after trypsinization was not investigated. It is of interest to note, however, that dark center particles were seen in untreated, ether and SDS, treatments.

The role of surface projections in HA remains a mystery, since they were not observed in any thin sections of the virus. Evidence from this study, although too subjective for quantitative analysis, suggests that trypsin-treated particles possess fewer projections. Therefore, their removal may expose a hemagglutinin between projections, or alteration of the surface itself may be necessary for HA to occur. Evidence that homotypic antisera inhibit HA (Nazerian, 1960) and attach solely to IBV surface projections (Berry and Almeida, 1968), while heterotypic (rabbit) antisera also inhibit HA (Biswal, 1963) but attach both to viral surface projections and envelopes, supports either opinion.

Conclusive evidence that trypsinized IBV is the hemagglutinin, or one of many hemagglutinins, has not been established. The reaction may result from a multitude of factors, all possibly interrelated. While "virus-like" particles were attached to erythrocytes, and were

contained in bridges annexing adjacent erythrocytes, bridge components may also contain extraneous AF material, similar in structure to IBV, solely, or in conjunction with altered trypsinized IBV.

Two hemagglutinins associated with poxviruses are not virions, but rather cellular and viral, phospholipid containing by-products of viral replication (Burnet, 1946; Chu, 1948; Gillen *et al.*, 1950; Youngner and Rubenstein, 1962), which can be separated from the virus by DEAE chromatography (McCrea and O'Laughlin, 1959).

Biswal *et al.* (1966) isolated noninfectious hemagglutinins, by DEAE column chromatography, which bridges adjacent erythrocytes, and were physically and chemically different from either treated or nontreated IBV.

Destruction of HA inhibitors, contained in AF, by trypsin, as opposed to a direct effect upon IBV or other hemagglutinins has also been suggested (Nazerian, 1965).

The formation of RBC stratum in ionagar suggests the strength of the hemagglutinin-erythrocyte bond. In a typical HA pattern, one could envision particles weakly attached to erythrocytes and physically obstructing RBC from rolling towards the center of the test tube, while securing of RBC in three dimensions would require a stronger, possibly electrostatic, bond.

Use of scanning electron microscopy in investigating virus-erythrocyte interactions has not been established (Carteaud *et al.*, 1973; Pirtle *et al.*, 1972). Although

protrusions from erythrocyte surfaces, and bridging of adjacent RBC after fixation with glutaraldehyde have been cited, the identity of such protrusions has not been elucidated. Improved technology where viral particles are recognized as such, or the use of monospecific antibodies complexed with latex particles, are possible solutions.

SUMMARY

1. Virion structural integrity was maintained during various conditions of temperature and preparation.

2. Surface projections were successfully fastened to virions by mild fixation with glutaraldehyde at host temperatures.

3. A 1% solution of PTA (pH 6.6) was the most effective stain. Penetration between surface projections and good contrast were its greatest advantages.

4. Trypsin affected partially purified preparations of IBV. A greater preponderance of "dark center" virions, "confirmed" or "suspected", was seen in treated samples.

5. Hemagglutination occurred in trypsinized, partially purified samples.

6. Virus-like particles were attached to chicken erythrocytes recovered from HA patterns. Virus-erythrocyte interactions occurred in both treated and nontreated samples; however, the interaction in the latter may have been produced by glutaraldehyde, and thus have been non-specific.

7. Immobilizing virus-erythrocyte interactions in ionagar prevented nonspecific bonds by glutaraldehyde.

Virus-like particles in ionagar were seen in bridges annexing adjacent erythrocytes.

8. Hemagglutination may result from a multitude of factors, all possibly interrelated. Hemagglutinins could be chemically and/or physically modified virions, modified or nonmodified extraneous AF material, or both.

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